

## FISH Analysis

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### 1. Introduction

#### 1.1. History and Principles

*In situ* hybridization of specific DNA or RNA sequences to cellular targets was developed over 20 yr ago (1,2). The early techniques employed isotopically labeled probes and subsequent autoradiographic detection using a photographic emulsion overlying the metaphase chromosomes, nuclei, or whole cells. However, autoradiography requires long exposure periods, and is not practical for clinical application. In the late 1970s, nonisotopic methods of nucleic acid labeling were developed. The subsequent improvements in the detection of reporter molecules using immunocytochemistry and immunofluorescence, in conjunction with advances in fluorescence microscopy and image analysis, have made the technique safer, faster and reliable.

During the past few years fluorescence *in situ* hybridization (FISH) has emerged as an extremely important tool for both basic and clinical research and application. This chapter focuses on FISH with DNA probes only. FISH is a technique that allows DNA sequences to be detected on metaphase chromosomes and interphase nuclei in tissue sections by using DNA probes specific for entire chromosomes or single unique sequences/genes. The steps of a FISH procedure are summarized in **Fig. 1**. In general, a specimen is treated with heat and formamide to denature the double-stranded DNA to become single stranded. The target DNA is then available for binding to a DNA probe with a complementary sequence that is also similarly denatured and single stranded. The probe and target DNA then hybridize to each other in a duplex based on complementary base pairing. The probe DNA is tagged with a hapten (such as biotin or digoxigenin) or is directly labeled with a fluorescent dye. Detection



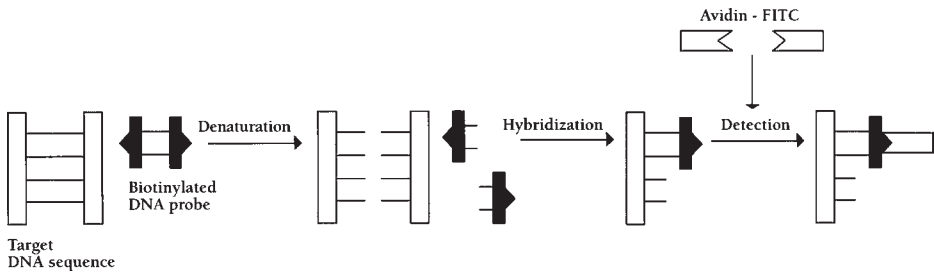


Fig. 1. Schematic presentation of some of the essential steps involved in FISH analysis.

of the hapten can be achieved with the application of an antibody tagged with a fluorescent dye (such as fluorescein, rhodamine, or Texas Red). Hybridization signals on a target material can be visualized through the use of a fluorescence microscope (3).

### 1.2. Comparison of FISH vs Conventional Cytogenetics

A large number of acquired chromosome changes have been reported in hematological malignancies that correlate with specific clinical, morphologic, and immunophenotypic features (4,5). Cytogenetic analysis is, therefore, a powerful tool in the assessment of these conditions. However, cytogenetic analysis alone is sometimes not sufficient to detect the chromosomal changes due to the fact that cytogenetic analysis can be performed only on dividing cells and the limitation of cytogenetic methods in some cases in which the abnormality is not visible with a conventional optic microscope.

FISH studies of the organization and function of chromosomal nucleic acid sequences have made it possible to gain information about chromosome changes in cells that are not in division, extending the possibilities of detecting anomalies not otherwise visible (particularly when only numerical chromosomal changes are to be ascertained). FISH is gaining increasing popularity, particularly because in addition to being an easy procedure for the detection of specific sequences in interphase or metaphase cells, it can also be applied to fixed and paraffin embedded tissues (6–8). However, FISH approaches also suffer from the shortcoming of the investigator having to know a priori which probes are to be used in each case being examined. The use of FISH based on painting and cosmid probes also requires knowledge regarding the exact anomalies to be ascertained. Detection sensitivity for FISH and other techniques is shown in **Table 1**.



**Table 1**  
**Comparison of FISH with Other Assays**

Technique	Marker	Detection limits
Routine pathology	Cellular morphology	$10^{-1}$ – $10^{-2}$
Cytogenetics	Chromosome morphology	$10^{-1}$ – $10^{-2}$
FISH	Chromosome structure	$10^{-2}$
Gene rearrangement	DNA configuration	$10^{-2}$ – $10^{-3}$
FACS analysis	Antigen profile	$10^{-3}$
Clonogenic culture	In vitro growth	$10^{-5}$
PCR	DNA/RNA structure	$10^{-5}$

FACS, fluorescence activated cell sorting; PCR, polymerase chain reaction.

**1.3. Clinical Applications of FISH in Hematologic Malignancies**

During the last decade there has been an exponential increase in the application of FISH techniques to various facets of human genetics (7–11). The rapid advances in the human genome effort, and the continuing elucidation of the genetic pathways of human diseases, have yielded readily available nucleic acid reagents required for the clinical application of FISH technology. FISH has been widely used to study the genetic events underlying hematopoietic disorders and to classify these disorders in a meaningful way, as well as to monitor the response to various therapeutic interventions (*see Table 2*). Both numerical and structural chromosome abnormalities are amenable to FISH analysis. A brief overview of examples of application of FISH in the study of hematologic disorders is given here.

**1.3.1. Acute Lymphoblastic Leukemia (ALL)**

Hyperdiploidy is found in 16–23% of adults and in up to 40% of children with ALL. The favorable prognosis associated with high hyperdiploidy (51–68 chromosomes) in children and adults with ALL is well established. FISH has been reported to potentially detect these cases with aneuploidy. Utilizing probes for 10 chromosomes (X, 4, 6, 8, 10, 14, 16, 18, 20, and 21), in particular combinations and in a stepwise manner, Moorman et al. (9) detected hyperdiploidy with FISH techniques in 94% of such cases and gave an accurate prediction of ploidy subgroups in 96% of these cases in a model population of 252 ALL cases. Our observations are also compatible with these findings. Therefore, these approaches may identify missing or hidden hyperdiploid cases among cases that have not been successfully analyzed cytogenetically.



**Table 2**  
**FISH Applications in Hematologic Disorders**

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Detection of diagnostic numerical and structural anomalies
Marker chromosome identification
Detection of gene amplification
Analysis of terminally differentiated or nondividing cells
Analysis of fixed or nonviable cells
Monitoring course of disease
Monitoring effects of therapy
Identification of the origin of a graft postallogenic bone marrow transplantation

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### *1.3.2. Acute and Chronic Myeloid Malignancies*

FISH, utilizing centromeric and unique sequence probes, has cogent and practical application in myeloid malignancies, including acute nonlymphocytic leukemia (ANLL), chronic myelocytic leukemia (CML), myeloproliferative disorders (MPD), and myelodysplastic syndromes (MDS), where it can be used to characterize these disorders, e.g., monosomy 7 (–7) and trisomy 8 (+8) in MDS, +8 and +9 in MPD, t(9;22) in CML, and t(15;17) in ANLL (11,12).

1. Anomalies of 11q23: Reciprocal translocations involving chromosome 11 at band q23 have been observed in both ALL and ANLL. The incidence of 11q23 abnormalities has been estimated to be approximately 5% in adult and childhood ALL and 75% or greater in infant leukemias. By FISH it was shown that most 11q23 rearrangements involve the same breakpoint cluster region of the MLL gene, although heterogeneity in the breakpoints in some of the rare rearrangements exists (13).
2. FISH in combination with morphology (MGG/FISH) was also used to detect minimal residual disease (MRD) in complete remission (CR) in leukemia patients with numerical chromosome aberrations at diagnosis. The results indicate that MGG/FISH may be a clinically useful method to detect MRD in acute leukemia and predict relapse, particularly when repeat studies are performed during CR (14).

### *1.3.3. Chronic Lymphocytic Leukemia (CLL)*

Chromosomal abnormalities have been described in about 50% of CLL patients using conventional cytogenetic methodologies. The most common abnormalities are trisomy 12 (+12) in 10–18% of cases and structural abnormalities of 13q14 in 10–28% of cases. However, accurate and successful cytogenetic analysis of specimens has been hindered by the low in vitro mitotic activity of the critical cell population and culture failure in up to 40% of the cases of CLL studied. Analysis of interphase cells provides a sensitive tool for



the detection of numerical cytogenetic abnormalities in poorly dividing cells. With FISH techniques, trisomy 12 has been reported in up to 63% of CLL cases (15).

Molecular studies, including FISH, have also demonstrated allelic deletion of the RB1 gene in 21–30% of CLL cases, and of the D13S25 marker, one megabase (Mb) telomeric to RB1, in 24–60% of the cases. Recently, FISH studies have provided further evidence for the existence of a new tumor suppressor locus in B-cell CLL located at 13q12.3 (16). BRCA2, located within the minimal deletion consensus, is a candidate for the gene. Interestingly, in most conventional chromosome banding studies of B-CLL, 11q deletions have not been identified as a frequent change. However, with FISH using the yeast artificial chromosome (YAC) clone 755b11 from the chromosome region 11q22.3-23.1, 11q deletions (20%) were found to be the second most frequent chromosome aberration following 13q14 deletions (17).

#### 1.3.4. Lymphoma

The most common characteristic chromosome abnormalities in B-cell non-Hodgkin's lymphoma (NHL) are translocations involving 14q32, such as t(8;14)(q24;q32) in Burkitt's lymphoma, t(14;18)(q32;q21) in follicular NHL, t(11;14)(q13;q32) in intermediate lymphocytic lymphoma/mantle-cell lymphoma, and t(3;14)(q27;q32) in diffuse lymphomas with large-cell components. However, cytogenetic investigations are not always successful in lymphoma, due to poor or lack of metaphase spreads and suboptimal chromosomal morphology. Recently, it was reported that a set of probes for interphase FISH analysis has been successfully established for the detection of tumor-specific rearrangements of the immunoglobulin heavy-chain (IgH) gene in B-cell malignancies (18). The results indicate that interphase FISH with IgH gene probes may be a rapid and reliable method to identify lymphoma-related gene rearrangements. As mentioned before, 50–75% of mantle-cell lymphomas (MCL) are associated with the t(11;14)(q13;q32). Using Southern blot analysis, a BCL1 breakpoint can be detected in about 50% of MCL cases. Utilizing FISH with two probe sets of differently labeled cosmids, symmetrically localized at either side of the major translation cluster of BCL1, it was reported that this FISH approach can be used to distinguish the t(11;14) from other 11q13 rearrangements in hematologic malignancies (19). Following the same strategy, the t(2;5)(p23;q35), that occurs in 25–30% of anaplastic large-cell lymphoma, was also reported to be successfully detected by interphase FISH (20). Furthermore, numerical chromosomal abnormalities in NHL were also investigated with interphase FISH. One study indicated that trisomy 12 (+12) was detected in 33% of the patients with follicular lymphoma, polysomy 12 in 37% of patients with diffuse large-cell lymphoma, monosomy 18 in 43% of



cases with CLL, and 28% of those with small-cell lymphocytic lymphoma, trisomy, or tetrasomy 17 in 27% of NHL patients, and X-chromosome aneuploidy in patients with NHL (21).

## **2. Materials**

### **2.1. Specimens**

Due to the high stability of DNA, FISH can be performed on most specimens, ranging from blood and bone marrow smears, buccal smears, cytopspins, and touch print preparations to archival pathology specimens and epithelial cells in bladder washings and urine. Logically, any nucleus can be evaluated with FISH methods as long as the DNA in the cell is not degraded (*see Note 1*).

For hematological disorders, bone marrow (BM) and peripheral blood (PB) are usually the specimens submitted for FISH analysis. Often these samples are first processed for chromosomal analysis and FISH is performed on the remaining fixed-cell pellet in cases of unsuccessful cytogenetics, to optimally interpret the observed abnormality or as a monitoring tool during treatment. BM is an ideal tissue for the observation of the *in vivo* chromosomal situation. Unstimulated blood cells are examined in order to observe the leukemic cells spontaneously dividing in the PB. Stimulated PB cells are used to examine T-cell or B-cell types that may be involved in specific lymphocytic diseases.

BM and PB smears also can be used for rapid FISH analysis. Lysis of red blood cells and fixation of cells can be accomplished after the smear is made. Generally, this kind of preparation can be employed with any DNA probe.

### **2.2. DNA Probes**

Three major categories of DNA sequences are used for probes in FISH analysis.

#### **2.2.1. Centromere-Specific Alpha Satellite DNA Sequence Probes**

The most popular type of probe consists of the chromosome centromere-specific alpha satellite DNA sequences which have a 171-bp DNA monomer, and are tandem repetitive and polymorphic, and do not code for a gene product (22). The alpha satellite DNA is almost identical in all human chromosomes except for 2–3% of the DNA, which is variable to the degree that centromeres of each individual chromosome can be distinguished and probes to these chromosomes can be generated (23). Other repetitive DNA sequence probes include those produced from the beta satellite DNA, which consists of a 68-bp monomer arranged in the same fashion as the alpha satellite DNA and is located at the tip of each acrocentric chromosome (24), as well as the classical satellite I DNA, which is an AATGG repeat found on chromosomes 1, 9, 15,



16, and Y (25). The major use of these satellite DNA probes is in the rapid enumeration of chromosomal monosomies or trisomies (*see Fig. 2*). Because the targets are large and repeated many times, these probes generate large signals.

### 2.2.2. Sequence-Specific Sequence Probes

The detection of unique single-copy genes (*see Fig. 3*) is accomplished by the use of sequence-specific probes. Levels of detection range from sequences as small as 1 kb up to as large as 300 kb (26). The various FISH unique-sequence probes are usually employed to detect microdeletion syndromes and rearrangements of oncogenes. Subtelomeric probes are produced from unique sequences in close proximity to the ends of chromosomes and are often used for the analysis of cryptic translocations.

### 2.2.3. Whole Chromosome and Arm-Specific Sequence Probes

Whole chromosome probes (WCP) and chromosome arm-specific probes consist of numerous unique and repetitive sequences from an entire or a partial chromosome. They can be derived from somatic cell hybrids; single flow sorted chromosomes, or microdissection of specific chromosomes with PCR amplification of the dissected DNA (27,28). These probes are primarily designed for application on metaphase chromosomes in analysis of markers and complex chromosomal rearrangements.

## 2.3. Probe Labeling

*In situ* hybridization was successfully performed in the past with the use of light microscopic detection methods utilizing horseradish peroxidase and other immunocytochemical reagents. However, except for the alpha satellite probes, unique sequence *in situ* hybridization probes cannot be easily resolved using a light microscope. FISH probes are more readily visualized with fluorescence microscopy.

Direct and indirect procedures are the two types of commonly used nonradioactive hybridization methods. Incorporation of nonisotopic reporter molecules into probes is achieved enzymatically or chemically (29). In the direct procedure, probe nucleotides are directly labeled with fluorochromes. The bound probe and target can be visualized directly with fluorescence microscopy. Incorporation of fluorochromes into probes can be accomplished with the use of polymerase enzymes and labeled nucleoside triphosphates (30). In the indirect method, DNA probes are tagged with a hapten, the most commonly used being biotin or digoxigenin. Biotin binds to avidin or streptavidin with high affinity and is used for the detection of biotin-labeled probes. Antibodies to digoxigenin are used for the detection of digoxigenin-labeled probes.



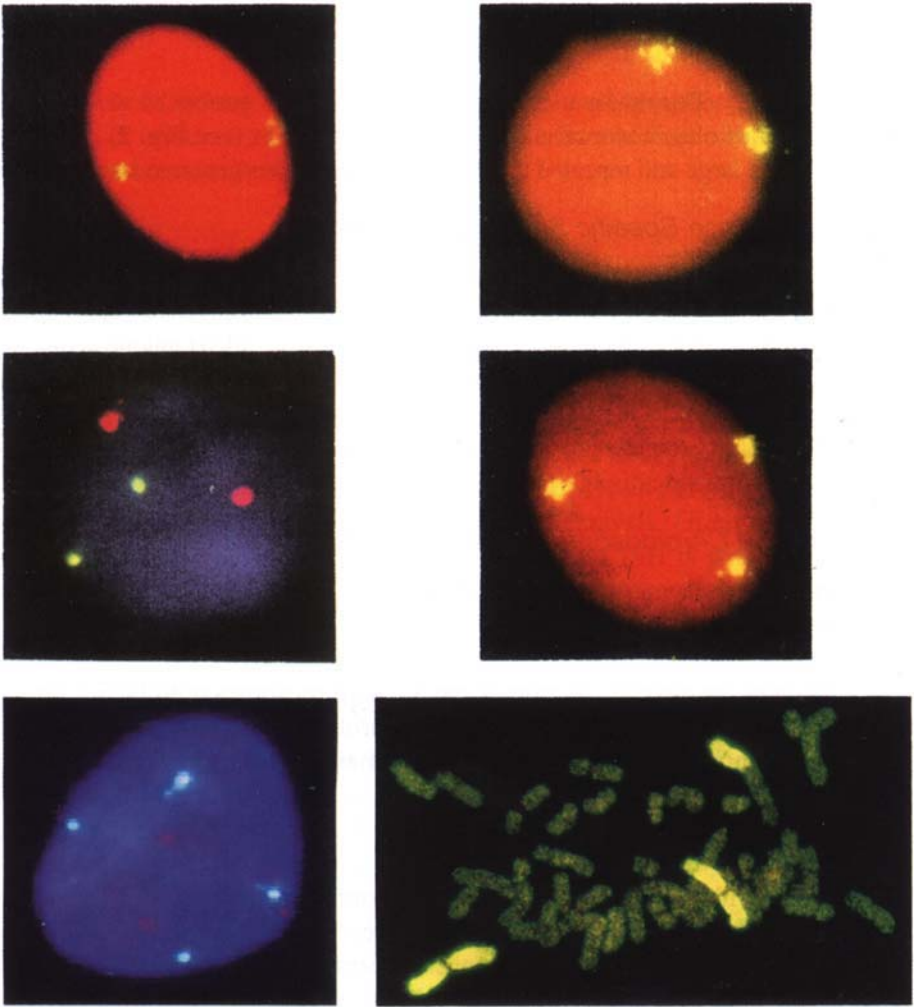


Fig. 2. In this plate are shown some representative results obtained with FISH. (upper left) Two signals obtained with a centromeric probe for chromosome 7 in a normal interphase cell. (upper right) Two signals obtained with a centromeric probe for chromosome 8 in a normal interphase cell. (middle left) Signals obtained when two differently labeled probes (chromosomes 7 and 9) were applied to a normal interphase cell. The red signals are those of chromosome 7 and the yellow-green for chromosome 9. (middle right) A leukemic interphase marrow cell showing three signals for the centromeric probe of chromosome 8. This finding indicates trisomy 8 (+8) to be present. (lower left) An interphase cell from a case with myelodysplastic syndrome showing three red signals for a centromeric probe for chromosome 8 (trisomy 8) and four blue signals for the probe for chromosome 10 (tetrasomy 10). (lower right) FISH using chromosome painting for chromosome 1 in a bone marrow metaphase. Two normal chromosomes 1 are present, as well as a derivative chromosome (upper right of metaphase) containing one of the arms of a chromosome 1.



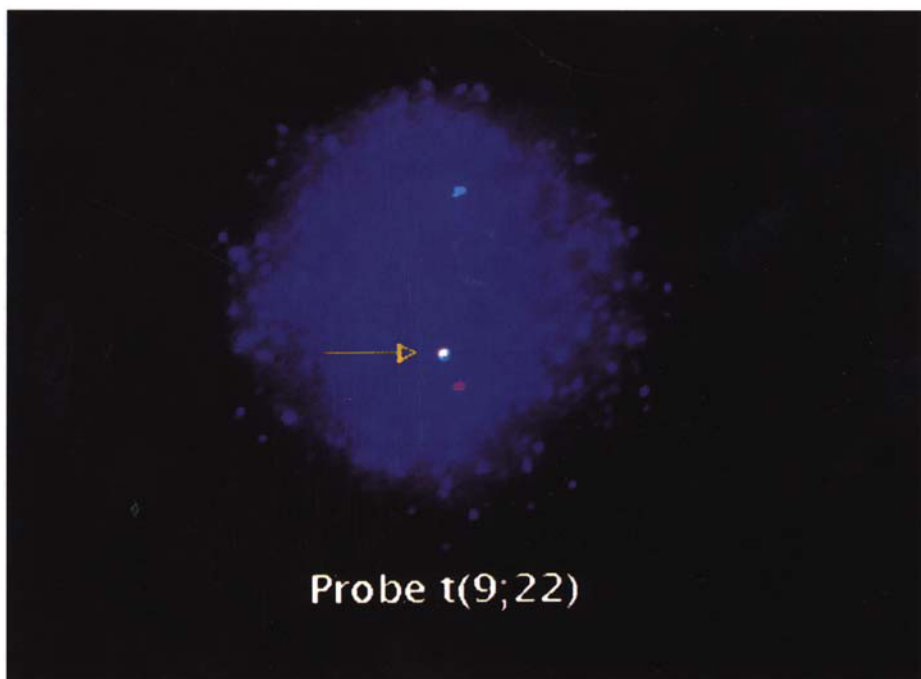


Fig. 3. Demonstration with FISH of the fusion product of the Ph translocation,  $t(9;22)(q34;q11)$  using a cosmid probe (arrow) for the translocation product.

Where a hapten is used as the reporter molecule, labeling methods include nick translation (31), random priming (32), in vitro transcription (33), and PCR amplification (34).

Multicolor labeling and detection have also gained popularity due to the flexibility of using labeling reagents. Two or three distinguishable colors can be visualized concurrently to study various targets of interest.

Counterstains help visualize the surrounding DNA or background nuclear material, the commonly used ones being propidium iodide (PI) and diamidino-2-phenylindole (DAPI). Both are DNA intercalators and fluorescent under similar wavelengths as are other fluorochromes, such as fluorescein, Texas Red, rhodamine, spectrum orange, and spectrum green. In general, when using a red fluorochrome, such as Texas Red or rhodamine or a dual-labeling study, such as a red and green/yellow dye analysis, the blue DAPI counterstain is the ideal choice; when using a green fluorochrome, such as fluorescein or spectrum green, PI counterstain is the best. An antibleaching chemical is commonly used to preserve the signal during storage and photography. Fading of fluorochromes on excitation is a photochemical process. Mounting media containing



diphenylene diamine or other agents act as radical scavengers and antioxidants that alleviate the quenching without altering the experimental results.

## **2.4. Reagents**

### **2.4.1. Prehybridization and Hybridization**

1. Herring sperm DNA (0.5 mg/mL).
2. RNase (1.0 mg/mL).
3. 70, 85, and 100% ice-cold ethanol.
4. Denaturing solution: 70% formamide, 2X standard saline citrate (SSC) (pH 7.0).
5. Hybridization master mix (MM 2.1): 55% formamide, 10% dextran sulfate, 1X SSC.
6. Rubber cement, prepared slides, and coverslips.

### **2.4.2 Posthybridization Washes and Signal Detection**

1. Wash solutions (stringency-dependent on type of probes): 50% formamide, 2X SSC (pH 7.0).
2. PN buffer: 0.1 M NaPO<sub>4</sub> (pH 8.0), 0.1% Nonidet P-40 (NP-40).
3. PNM buffer: NaAzide in PN (0.2 mg/mL).
4. 2X SSC (pH 7.0).
5. 2X SSC (pH 7.0), 0.1% NP-40.
6. 0.1X SSC (pH 7.0).
7. Antidigoxigenin-fluorescein isothiocyanate (FITC) (20 µg/mL in PNM or Oncor), or-rhodamine (Oncor).
8. Rabbit anti-sheep antibody I (Oncor).
9. Anti-rabbit antibody II-FITC (Oncor) or rhodamine (Oncor).
10. Avidin-FITC (5 µg/mL in PNM or Oncor).
11. Biotinylated anti-avidin (5 µg/mL in PNM or Oncor).
12. Antifade solution: P-phenylenediamine dihydrochloride in PBS (10 mg/mL).
13. PI (1 µg/mL).
14. DAPI (1 µg/mL).
15. Mounting medium: antifade solution, counterstain, i.e., PI or DAPI (self-made or Oncor or Vysis).

## **2.5. Instruments**

- |                 |                          |
|-----------------|--------------------------|
| 1. Centrifuge   | 7. Slide warmer          |
| 2. Thermometers | 8. Refrigerator, freezer |
| 3. Timers       | 9. pH meter              |
| 4. Pipetman     | 10. Balance              |
| 5. Waterbaths   | 11. Forceps              |
| 6. Incubator    |                          |



### 3. Methods

#### 3.1. Slide Preparation

##### 3.1.1. Fixed Cell Pellet

Metaphase or interphase cell slides are prepared from fixed BM or PB cell suspensions by conventional cytogenetic techniques in such a way that most of the cytoplasm is not visible around the metaphases and nuclei. Slides are air-dried for 10 min to overnight. Baking the slides in an oven is not recommended. Until FISH can be performed, the slides are stored in 70% ethanol at 4°C for a minimum of 2 h to a maximum of 2 wk. Best results are achieved when slides are used within the first 2 wk.

Fresh slides can be used without pretreatment. Slides older than a week should be pretreated as follows:

1. Incubate in RNase A (1.0 mg/mL) for 1/2–1 h at 37°C.
2. If a heavy cytoplasm is present, treat the slides further in 100% acetic acid for 1/2 h.
3. Dehydrate the slides in 70, 85, and 100% ethanol series for 1 min each at room temperature. Air-dry.

##### 3.1.2. BM and PB Smears

1. Allow freshly made smear slides to air-dry for 10–30 min at room temperature.
2. Fix the slides for 5 min in 100% methanol. Air-dry.
3. Apply 50  $\mu$ L of RNase A (1.0 mg/mL) solution onto each slide, adding a 25  $\times$  25mm glass coverslip and incubate it at 37°C for 30 min.
4. Rinse the slides with distilled water.
5. Place the slides in 2X SSC at 37°C for 30 min.
6. Place the slides in 70, 85, and 100% ethanol series at room temperature for 2 min each.
7. Air-dry. Subsequently perform a FISH analysis.

##### 3.1.3. Giemsa-Pretreated Slides

1. Fix slides for 15–20 min in 100% methanol two times.
2. Place the slides in 70, 85, and 100% ethanol series at room temperature for 1 min each.
3. Place the slides in 3:1 methanol:glacial acetic acid for 10 min. Air-dry.
4. Wash the slides in 3.7% formaldehyde for 10 min and phosphate-buffered saline (PBS) for 5 min two times.
5. Place the slides in 70, 85, and 100% ethanol series at room temperature for 1 min each. Air-dry. Subsequently perform a FISH analysis.



### 3.2. FISH Procedures

Probes available commercially are accompanied by the manufacturer's suggestions on probe preparation and hybridization. Detection kits are also available from various manufacturers. Presented here are the procedures we use in our laboratory, which were established on the basis of the literature (3,35) and some of the commercial manufacturers' guidelines (Vysis, Oncor) with modifications. The procedures for directly labeled probes are primarily based on Vysis procedure guidelines.

#### 3.2.1 Repetitive Sequences Probes

##### 3.2.1.1. INDIRECTLY (DIGOXIGENIN OR BIOTIN)-LABELED PROBES

###### 1. Denaturation and Hybridization:

- a. Use a previously refrigerated Coplin jar containing denaturing solution and place it in a waterbath. Turn on the waterbath and bring the temperature up to 70–72°C inside the Coplin jar. Before hybridization place a clean thermometer into the Coplin jar to check the exact temperature of the hybridization solution. The denaturing temperature of 70°C is critical, and each slide that is placed into the solution will drop the temperature one degree. Denature no more than two slides at a time. If the waterbath has been turned on and is already up to the temperature, place denaturing solution in a 37°C waterbath for 10 min, then in a 65°C waterbath for 10 min, and finally in a 72°C waterbath.
- b. Turn on a warming tray to 40°C and wash off the surface with 70% ethanol.
- c. Use a cold ethanol series (70%, 85%, 100%) previously kept in a freezer. Do this just before starting to denature slides.
- d. Place slides in the denaturing solution for 2 min.
- e. Dehydrate the slides in the cold ethanol series (70%, 85%, 100%) for 2 min each, with some agitation.
- f. Air-dry slides.
- g. Label probe vials with the probe to be made.
- h. Prepare probe mixtures. For each 22 × 22 mm coverslip, use 10 µL of probe mixture. A 22 × 50 mm coverslip requires 20 µL of probe mixture.
  - i. 7 µL MM 2.1
  - ii. 1 µL carrier DNA (0.5 mg/mL)—herring sperm
  - iii. 2 µL probe DNA (0.5 mg/mL)
- i. Vortex probe vials and microfuge for a short time to mix and concentrate probe mixtures in the bottom of vials.
- j. Use a microtube floating rack, float probe mixtures in a 72°C waterbath for 5 min.
- k. Immediately chill probe mixtures in a freezer for approximately 2 min.
  1. Vortex and microfuge to collect all droplets.
- m. Place the air-dried slides and probe mixtures on a warming tray. Bring slides, probe mixtures, coverslips, pipet tips and moist chambers to approximately 37–40°C.



- n. Pipet probe mixture onto each slide and add a coverslip, trying to avoid the formation of air bubbles. If there are air bubbles under a coverslip, press the coverslip with forceps and work bubbles to the sides of the coverslip. Once the probe is on a slide, the temperature should never be allowed to drop below 37°C, as this can cause nonspecific binding which will not wash off (*see Note 2*).
  - o. Use a 10-cm<sup>3</sup> syringe to seal edges of coverslips with rubber cement.
  - p. Place the slides in warm, moist chambers and place these into a 37°C incubator overnight.
2. Post-hybridization wash:
- a. Turn on a warming tray to 40°C.
  - b. Remove three wash jars from refrigerator and place them in order in a cool waterbath. (Placing cold jars into a hot bath may cause the jars to shatter.) Turn on the waterbath to an appropriate washing temperature. Allow approximately 30 min for the waterbath to equilibrate. The recommended temperature is good for two slides; temperature has to be adjusted to accommodate more slides (0.5°C higher per slide added).
  - c. Remove moist chambers from the incubator. Place chambers on the warming tray (40°C).
  - d. Peel off rubber cement with forceps. Place slides in the first wash solution, let sit for a minute or so, and then remove coverslips with forceps. Coverslips should just slide off without difficulty. If tension persists, let them sit in the wash solution a little longer. DO NOT pull up coverslips, as this will damage the cells. Once the coverslip is off, agitate slide(s) and incubate for 2 min.
  - e. Wash the slides in washes 2 and 3, respectively, for 2 min each, with agitation. Always use jars in the same order.
  - f. Place the slides in 2X SSC (pH 7.0) at room temperature for 2 min.
  - g. Follow with 2 min (minimum time) wash in PN buffer at room temperature. Slide(s) can remain in PN buffer for hours at room temperature or even overnight at 4°C before proceeding to the next step.
3. Detection (for digoxigenin-labeled probes):
- a. Remove the slides from PN buffer and blot excess fluid from the edge. Do not allow the slide surface to dry; this will cause nonspecific binding of the detection reagent and high background fluorescence (*see Note 3*).
  - b. Apply 30 µl of fluorescein-labeled anti-digoxigenin or rhodamine-labeled anti-digoxigenin to each slide and place a plastic coverslip over the solution. Incubate the slides at 37°C for 5 min in prewarmed humidified chambers.
  - c. Take the humidified chambers out of the incubator and place them on a warming tray (37°C). Dip the slides in PN buffer to remove coverslips.
  - d. Wash the slides three times for 2 min each in 40 ml of PN buffer at room temperature. These washes remove excess detection compounds.
4. Digoxigenin Amplification
- a. Remove the slides from PN buffer and blot excess fluid from the edge. Do not allow slide surface to dry.



- b. Apply 20  $\mu\text{L}$  of rabbit anti-sheep antibody I to each slide and place a plastic coverslip over the solution. Incubate the slides at 37°C for 15 min in a prewarmed humidified chamber.
  - c. Dip the slides in PN buffer to remove coverslips. Wash the slides three times for 2 min each in 40 mL of PN buffer at room temperature.
  - d. Apply 20  $\mu\text{L}$  of fluorescein or rhodamine-labeled anti-rabbit antibody II to each slide and place a plastic coverslip over the solution. Incubate the slides at 37°C for 15 min in a prewarmed humidified chamber.
  - e. Dip the slides in PN buffer to remove coverslips. Wash the slides three times for 2 min each in 40 mL of PN buffer at room temperature.
  - f. Counterstain with 10  $\mu\text{L}$  mounting medium. Place a glass coverslip on each slide, remove any bubbles, and blot excess PI or DAPI by placing the slide between two pieces of bibulous paper and pressing on the slide. View with a fluorescent microscope (*see Note 4*).
  - g. Keep slides in a light-tight box until they are scored. They can be kept at 4°C for 7–10 d.
5. Detection (for biotin-labeled probe):
    - a. Take the slides out of PN buffer and add 20  $\mu\text{L}$  of avidin to each slide, place a plastic coverslip over the solution, place the slides in moist chambers, and incubate them for 5 min in an incubator at 37°C.
    - b. Wash the slides three times in fresh PN buffer at room temperature for 2 min each with agitation. Coplin jars must be wrapped in foil. Signal will decrease with exposure to light. With some of the more repeated probes the signal may be visible at this point, however, we usually proceed with one round of amplification.
  6. Amplification for biotin-labeled probe:
    - a. Apply 20  $\mu\text{L}$  of anti-avidin to each slide. Place a plastic coverslip over the solution. Place the slides in moist chambers and incubate them at 37°C for 5 min.
    - b. Wash the slides three times in PN buffer at room temperature for 2 min each with agitation.
    - c. Apply 20  $\mu\text{L}$  of avidin to each slide, place a plastic coverslip over the solution. Place the slides in moist chambers and incubate them at 37°C for 5 min.
    - d. Wash the slides three times in fresh PN buffer at room temperature for 2 min each with agitation. (For very weak probes, **steps a–d** can be repeated to obtain a second round of amplification and therefore a brighter signal; however the background will also be increased, *see Note 5*.)
    - e. Drain excess fluid from the slides but do not allow the slides to dry. Pipet 10  $\mu\text{L}$  of PI/antifade or 10  $\mu\text{L}$  of DAPI for each 22  $\times$  22 mm coverslip onto the slide (20  $\mu\text{L}$  for a 22  $\times$  50 mm coverslip).

### 3.2.1.2. DIRECTLY-LABELED PROBES

1. Probe preparation:
  - a. At room temperature, mix 7  $\mu\text{L}$  of CEP hybridization buffer (Vysis), 1  $\mu\text{L}$  of directly labeled CEP DNA probe and 2  $\mu\text{L}$  sterile deionized water in a



microcentrifuge tube. For dual color, mix 7  $\mu\text{L}$  of CEP hybridization buffer, 1  $\mu\text{L}$  spectrum orange DNA probe, 1  $\mu\text{L}$  spectrum green DNA probe, and 1  $\mu\text{L}$  sterile deionized water.

- b. Centrifuge 1–3 seconds in a microcentrifuge.
2. Denaturation and hybridization:
  - a. Remove the Coplin jar containing denaturing solution from the refrigerator and place it in a 70–75°C waterbath, which has been turned off. Turn on the waterbath and bring temperature to 70–75°C. (Placing a cold Coplin jar in hot waterbath may cause the jar to shatter.)
  - b. Turn on a warming tray to 45°C.
  - c. Denature DNA probe mixture for 5 min in a 70–75°C waterbath.
  - d. Denature slides in the denaturing solution for 5 min.
  - e. Wash the slides 1 minute each in cold ethanol series (70%, 85%, and 100%).
  - f. Air-dry the slides.
  - g. Place slide pipet tips, the probe mixture, and coverslips on the slide warmer (45°C).
  - h. Pipet 10  $\mu\text{L}$  of probe mixture onto each slide, adding a coverslip, and seal edges with rubber cement.
  - i. Place the slides in humidified chambers and incubate them for 16–24 h (overnight) in a 37°C incubator.
3. Post-hybridization wash and detection:
  - a. Turn on a slide warmer to 45°C and place the humidified chambers on the slide warmer.
  - b. Place three wash solutions in a waterbath and bring the temperature up to 45°C.
  - c. Remove rubber cement and coverslips.
  - d. Wash the slides three times for 10 min each in 45°C wash solutions, keeping solutions in correct order. No more than two slides should be processed per wash procedure.
  - e. Wash the slides for 10 min in 2X SSC at 45°C.
  - f. Wash the slides for 5 min in 2X SSC/0.1% NP-40 (40  $\mu\text{L}$  NP-40 in 40 mL 2X SSC) at 45°C.
  - g. Allow the slides to air-dry in darkness.
  - h. Apply 10  $\mu\text{L}$  mounting medium (for two-color hybridization, DAPI counterstain works best) and a coverslip to each slide.
  - i. Store the slides in a light-tight box until they are ready to be scored. They can be kept at –20°C.

### 3.2.2. Unique Sequences Probes

#### 3.2.2.1. INDIRECTLY (DIGOXIGENIN OR BIOTIN)-LABELED PROBES

1. Slide pretreatment: Place slides in 2X SSC (pH 7.0) at 37°C for 30 min. Dehydrate the slides at room temperature in 70, 85, and 100% ethanol for 2 min each. Air-dry.



2. Slide denaturation: Denature the slides in denaturing solution at 70°C for 2 min; dehydrate the slides in cold 70, 85, and 100% ethanol series for 2 min each and air-dry.
3. Probe preparation and hybridization:
  - a. Prewarm probe mixture (Oncor) at 37°C for 5 min. DO NOT HEAT DENATURED PROBES.
  - b. Vortex the probe mixture and microfuge before pipetting.
  - c. Prewarm the slides in a humidified chamber at 37°C.
  - d. Apply 20  $\mu$ L of probe mixture per 22  $\times$  50 mm coverslip or 10  $\mu$ L of probe mixture per 22  $\times$  22 mm coverslip to each slide. Apply glass coverslips and seal with rubber cement. Incubate the slides for 16–24 h at 37°C in humidified chambers.
4. Posthybridization wash:
  - a. Use the series of three washes (50% formamide/2X SSC pH 7.0) for 5 min each at 43°C.
  - b. Place the slides in 2X SSC, pH 7.0 at 37°C for 8 min.
  - c. Transfer the slides to PN buffer for 2 min.
5. Detection: See the indirectly labeled repetitive sequences probe procedure in **Subheading 3.2.1.1.** for the digoxigenin-labeled or biotin-labeled detection and amplification.

#### 3.2.2.2. DIRECTLY LABELED PROBES

1. Slide pretreatment: The same procedure as for indirectly labeled unique sequences probes.
2. Probe preparation:
  - a. At room temperature, mix 7  $\mu$ L of large-scale integration (LSI) hybridization buffer (Vysis), 1  $\mu$ L of directly labeled cosmid DNA probe and 2  $\mu$ L sterile deionized water in a microcentrifuge tube.
  - b. Centrifuge 1–3 s in a microcentrifuge.
3. Denaturation and hybridization:
  - a. Denature DNA probe mixture for 5 minutes in a 70–75°C waterbath.
  - b. Denature slides in denaturing solution at 70–75°C for 5 min.
  - c. Wash the slides 1 min each in cold 70, 85, and 100% ethanol series.
  - d. Air-dry the slides.
  - e. Place slide pipet tips, the probe mixture, and coverslips on the slidewarmer (45°C).
  - f. Pipet 10  $\mu$ L of probe mixture onto each slide, adding a coverslip, and seal edges with rubber cement.
  - g. Place the slides in humidified chambers and incubate them for 16–24 h (overnight) in a 37°C incubator.
4. Post-hybridization wash and detection:
  - a. Wash the slides three times for 10 min each in 45°C wash solutions, keeping solutions in correct order. No more than two slides per wash procedure.
  - b. Wash the slides for 10 min in 2X SSC at 45°C.



- c. Wash the slides for 5 min in 2X SSC/0.1% NP-40 (40  $\mu$ L NP-40 in 40 mL 2X SSC) at 45°C.
- d. Allow the slides to air-dry in darkness.
- e. Apply 10  $\mu$ L mounting medium (for two-color hybridization, DAPI counter-stain works best) and a coverslip to each slide.
- f. Store the slides in a light-tight box until they are ready to be scored. They can be kept at -20°C.

### 3.2.3. Whole Chromosome Painting Probes

#### 3.2.3.1. INDIRECTLY (DIGOXIGENIN OR BIOTIN)-LABELED PROBES

1. Probe preparation:
  - a. Prewarm probe mixture (Oncor) at 37°C for 5 min.
  - b. Aliquot 10  $\mu$ L of the probe mixture into a microcentrifuge tube.
  - c. Denature the probe mixture at 70°C for 10 min.
  - d. Incubate the probe mixture at 37°C for 2 h to preanneal.
2. Denaturation and hybridization:
  - a. Denature slides in denaturing solution at 70°C for 2 min.
  - b. Dehydrate the slides in cold ethanol series (70, 85, 100%) for 2 min each, with some agitation.
  - c. Air-dry slides.
  - d. Pipet 10  $\mu$ L of probe mixture onto each slide, adding a coverslip, and seal edges with rubber cement.
  - e. Place the slides in humidified chambers and incubate for 16–24 h (overnight) in a 37°C incubator.
3. Posthybridization wash:
  - a. Wash the slides for 5 min in each of three washes (43°C).
  - b. Place the slides in 0.1X SSC, pH 7.0 at 60°C for 8 min.
  - c. Place the slides in PN buffer for 2 min.
4. Detection: See the indirectly labeled repetitive sequences probe procedure in **Subheading 3.2.1.1.** for digoxigenin-labeled or biotin-labeled detection and amplification.

#### 3.2.3.2. DIRECTLY LABELED PROBES

1. Probe preparation:
  - a. Allow whole chromosome painting (WCP) hybridization buffer (Vysis) to warm to room temperature so that its viscosity decreases to the point that it may be accurately pipetted.
  - b. In a microcentrifuge tube add 7  $\mu$ L WCP hybridization buffer, 1  $\mu$ L WCP DNA probe, and 2  $\mu$ L deionized water. This quantity of probe mixture is sufficient to cover one 22 mm  $\times$  22 mm hybridization zone.
  - c. To screen samples for two WCP probes simultaneously, prepare probe mixture as follows: 7  $\mu$ L WCP hybridization buffer, 1  $\mu$ L spectrum orange WCP DNA probe, 1  $\mu$ L spectrum green WCP DNA probe, and 1  $\mu$ L deionized water.



- d. DAPI counterstain must be used for visualization, as PI counterstain will fluoresce in the same region of the spectrum as the spectrum orange fluorophore.
- e. Denature the probe mixture for 5 min in a 73°C waterbath.
- f. Cool the probe mixture in a freezer for 1–2 min.
2. Denaturation and hybridization:
  - a. Immerse slides in 70–73°C denaturing solution for 5 min to denature the target DNA. To maintain the temperature of the denaturing solution, place no more than two slides in denaturing solution at one time. Longer or shorter denaturation time, for example 2–10 min, may be necessary for some specimens.
  - b. Dehydrate the slides at room temperature in 70, 85, and 100% ethanol wash solutions for 2 min each.
  - c. Place the slides on a 45°C slide warmer.
  - d. Leave the slides on the slide warmer and apply the aliquot of the probe mixture to the target area of each slide. Place a prewarmed glass coverslip over the probe mixture and seal the edges with rubber cement.
  - e. Place the slides in preheated humidified chambers.
  - f. Place the chambers in a 37°C incubator. Allow hybridization to proceed for at least 4 h or, preferably, overnight.
3. Posthybridization wash and detection:
  - a. Wash the slides for 10 min in each of three washes (44°C).
  - b. Wash the slides in a jar containing 2X SSC, pH 7.0, preheated to 44°C for 10 min.
  - c. Wash the slides in a jar containing 2X SSC/0.1% NP-40 preheated to 44°C for 5 min with agitation.
  - d. Air-dry the slides in darkness.
  - e. Apply 10  $\mu$ L of mounting medium to the target area of each slide. Place a coverslip over the counterstain.
  - f. Place the slides in black boxes: the slides are now light sensitive and signals will fade if exposed to light.

#### 3.2.4. Visualization—Image Recording

Blue, green, and UV filter sets (e.g., Zeiss filter sets: 01, 09, 15; Nikon filter sets: G-20, B-12, UV-10) with a good fluorescence microscope, such as Zeiss Axioplan, Zeiss Axiophot, and Nikon Microphot FX, are necessary elements for the visualization of FISH results. For DAPI/fluorescein, Ektachrome 160 tungsten film works well. Kodacolor 400 and Fujichrome 400 are the better choices for photographing the red and yellow of PI/fluorescein.

Digital imaging systems also are now widely used in FISH analysis. These apparatuses consist of a combination of microscope, camera, and computer with advanced software, allowing the recording of images electronically by using video or low-light cameras. These devices are particularly useful for detailed FISH analysis of small signals, from phage and cosmid probes, or YACs crossing translocation breakpoints (*see Note 6*).



### 3.3. Regulations, Controls, and Analysis

The American College of Medical Genetics (ACMG) has developed some policies and quality assurance guidelines for the clinical application of FISH (36). The Food and Drug Administration (FDA) has also approved several DNA FISH probes for clinical use. However, in general, FISH still is considered an investigational technique with conventional cytogenetic results ultimately serving as the primary diagnostic test.

Probe validation and controls for probes and types of specimens should be established when performing FISH analysis (36). Probe validation assures that probes employed will produce the most successful hybridization with the highest analytical specificity and sensitivity. Controls will provide essential information about the success of an experiment and the criteria to evaluate the results of FISH studies. Finally, clinical validation of FISH procedures and results is important, because it will afford laboratory workers the opportunity to gain appropriate experience in the performance of the test system.

Because there are numerous sources of variation in FISH data between laboratories, e.g., differences in preparing samples, probes employed, FISH procedures, experience, and subjective counting criteria between observers, it is important that analysis criteria for interphase and metaphase FISH should be established for each laboratory performing FISH utilizing various probes on different specimens. A case in point are the criteria for scoring interphase FISH, including at least two technologists scoring the same case, examination of a large number of cells, avoiding damaged and overlapping nuclei, as well as areas of the slide where hybridization is absent or suboptimal, focusing up and down on each nucleus and so on. In addition, reporting criteria of FISH results are also important, which should include the probes used, the source and identification of the probes used, the number of test and control cells scored and detailed, hybridization results, limitations of the assay, and following ISCN 1995 (37) for FISH nomenclature.

## 4. Notes

1. No signal with a probe that has performed well previously: This situation may be related to the probe, which has degraded because of improper handling and/or shipping. It is important that FISH probes be stored at  $-20^{\circ}\text{C}$  and handled with gloves and autoclaved pipet tips. A change in sample type and sample degradation may also influence probe signal intensity.
2. Cross-hybridization (nonspecific fluorescent signals): Because exact pairing of DNA sequences is achieved and maintained under certain conditions, more stringent reaction conditions may be necessary to reduce crosshybridization, which can be accomplished by increasing the temperature of hybridization and rinses,



increasing formamide concentration or decreasing the concentration of salts (e.g., SSC). For blocking nonspecific hybridization signals when painting probes are being used, Cot-1 fraction of total human genomic DNA is often successful.

3. Nonspecific background: Components of blocking agents are often responsible for these problems. Changing blocking components or detection systems or preparing fresh solutions are helpful in solving these problems.
4. Suboptimal signal intensity: Common problems may be related to the microscope, including either the bulb alignment or the filter sets. In addition, there are several strategies to maximize signal intensity.
  - a. Diluting the counterstain with antifade until it is just bright enough to scan with a low-power objective (e.g., PI: 0.3  $\mu\text{g}/\text{mL}$  and DAPI: 0.05  $\mu\text{g}/\text{mL}$ ).
  - b. The use of amplification as described in the **Subheading 3.2.1.1**.
  - c. Repeating the hybridization using a lower-stringency wash.
5. Cytoplasmic background: Increased cytoplasmic background can reduce probe nuclear penetration and cause suboptimal hybridization. These conditions can be improved when the slide preparations are pretreated with proteinase-K (e.g., 0.6 mg/ml in 20 mmol/L Tris HCl, 2 mmol/L CaCl<sub>2</sub>, pH 7.5 for 1–5 min at 40°–42°C) and/or RNase.
6. Other powerful cytogenetic techniques: Comparative genomic hybridization (CGH) is another new molecular cytogenetic technique that has recently been developed for detecting chromosomal imbalances in tumor genomes (38). CGH is based on two-color FISH. Equal amounts of differentially labeled tumor DNA and normal DNA are mixed together and hybridized, under conditions of Cot-1 DNA suppression, to normal metaphase spreads. In a single experiment, CGH identifies DNA gains and losses and maps these variations to metaphase chromosomes. DNA extracted from either fresh or frozen tissues, cell lines, as well as from formalin-fixed, paraffin-embedded samples is suitable for CGH (39). CGH becomes particularly advantageous when structural analysis of chromosomal changes in cancers are severely limited by their banding quality. Of note is that CGH is an effective screening method for describing and establishing a phenotype/genotype correlation in solid tumor progression. Several examples of chromosomal aberrations that define specific stages in tumor progression have already been established in brain, colon, prostate, cervix, and breast carcinogenesis (38).

Cancer cytogenetics is often hampered by low mitotic indices, poor quality metaphase spreads, and the presence of complex marker chromosomes. A newly developed technique—multicolor spectral karyotyping (SKY)—may have the ability to overcome these obstacles (*see Fig. 4*). This technique combines Fourier spectroscopy, charge-coupled device (CCD) imaging, and optical microscopy to measure chromosome-specific spectra after FISH with differentially labeled painting probes (40,41). This technique was reported to be in excellent agreement with results from previously performed FISH experiments and banding analysis. Currently, work is underway to generate a multicolor banding pattern (bar code) of the human chromosome complement by using chromosome arm- and band-specific painting probes in order to identify intrachromosomal anoma-



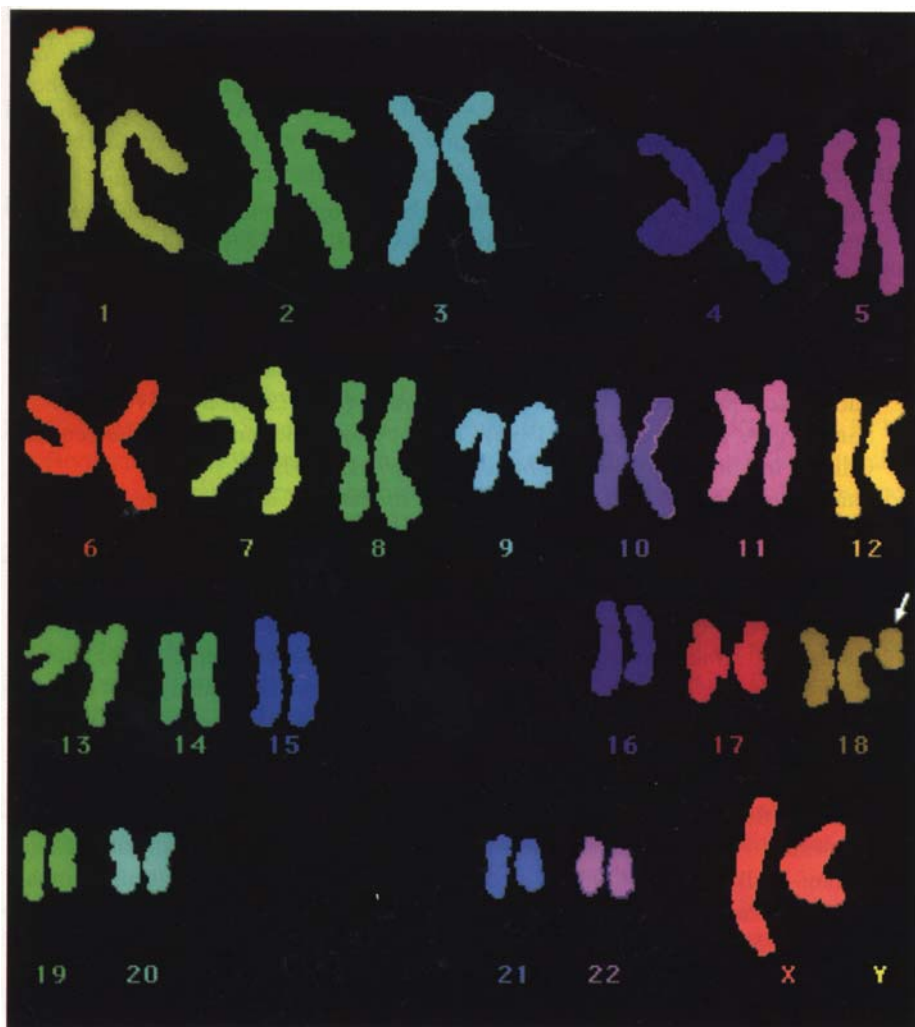


Fig. 4. A SKY picture showing the marker chromosome (indicated by arrow) to be of chromosome 18 in origin. This marker could not be identified with certainty by G-banding.

lies. Therefore, it appears that SKY may be a very promising approach to the rapid and automatic karyotyping of neoplastic cells.

Chromosomal microdissection to obtain DNA and subsequent PCR generation of FISH probes is another powerful analytical tool. Microdissected chromosomal DNA can generate whole chromosome paint probes and band- or region-specific probes. This technique is particularly useful in identifying the



origin of chromosomes or chromosomal regions that cannot be conclusively identified by cytogenetics. The approach, combining PCR to produce probes from microdissected chromosomal DNA and subsequent FISH analysis, has been defined as micro-FISH (42,43).

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