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New Approaches to Fluorescence *In Situ* Hybridization

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Summary

Fluorescence *in situ* hybridization (FISH) is a nonisotopic labeling and detection method that provides a direct way to determine the relative location or copy number of specific DNA sequences in nuclei or chromosomes. With recent advancements, this technique has found increased application in a number of research areas, including cytogenetics, prenatal diagnosis, cancer research and diagnosis, nuclear organization, gene loss and/or amplification, and gene mapping. The availability of different types of probe and the increasing number of FISH techniques has made it a widespread and diversely applied technology. Multicolor karyotyping by multicolor FISH and spectral karyotyping interphase FISH and comparative genomic hybridization allow genetic analysis of previously intractable targets. We present a brief overview of FISH technology and describe in detail methods of probe labeling and detection for different types of tissue sample, including microdissected nuclei from formalin-fixed paraffin-embedded tissue sections.

Key Words: Fluorescence *in situ* hybridization (FISH); interphase FISH; laser capture microdissection (LCM); LCM-FISH.

1. Introduction

Fluorescence *in situ* hybridization (FISH) is a powerful molecular–cytogenetic detection technique that utilizes a fluorescent-labeled DNA probe, which is hybridized to a genomic target in the nuclei of fixed cells, to ascertain the presence or absence of a particular DNA sequence. Hybridization to the target loci is visualized by the detection of fluorescent signals on metaphase chromosomes or interphase nuclei (*see* **Fig. 1**; Color Plate 11, following p. 274). Using appropriate probes, aberrations such as chromosomal aneuploidy, deletions, duplications, and translocations can be detected on a cell-to-cell basis in metaphase chromosome preparations or in nondividing interphase nuclei. Since the first demonstration of this fluorescence labeling technique ***(1–3)***, FISH has developed very rapidly to be a valuable tool in basic cytogenetic research and diagnosis. Advances in probe generation, hybridization technology, fluorescence

From: *Methods in Molecular Biology, vol. 319: Cell Imaging Techniques: Methods and Protocols*
Edited by: D. J. Taatjes and B. T. Mossman © Humana Press Inc., Totowa, NJ

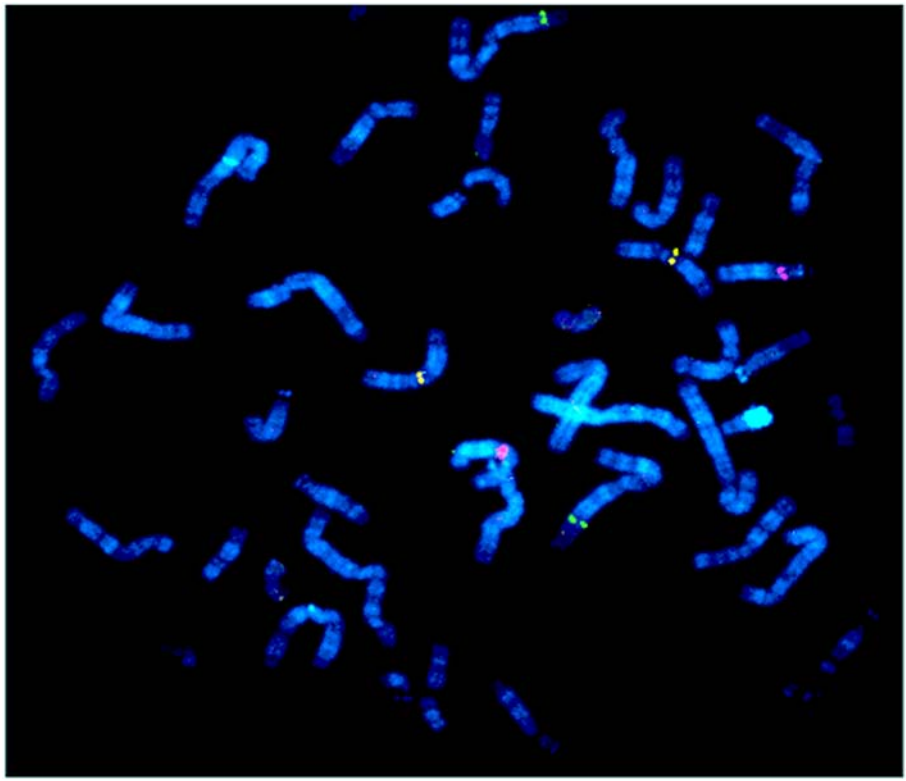

Fig. 1. Metaphase analysis with multiple single-gene probes. Multicolor FISH showing simultaneous localization of three human genomic BAC probes, CDKN2c (green), RB1 (red), and CCND1 (yellow), on a normal human metaphase spread. (*See* Color Plate 11, following p. 274.)

microscopy, and digital and spectral imaging have led to the rapid and improved development of molecular cytogenetic approaches that allow identification of chromosomal aberrations with unprecedented accuracy. Several reviews are available that emphasize the role of FISH in biological research as well as in genetic diagnostic applications ***(4–9)***.

1.1. FISH Probes

1.1.1. Centromeric Probes

Specific or restricted specificity alpha satellite probes are used to identify repeat sequence targets in the centromeric region of each chromosome. Chromosome-specific centromeric probes are now readily available commercially, enabling us to identify individual chromosomes and to analyze numerical chromosomal abnormalities in interphase and metaphase preparations.

1.1.2. Gene-Specific Probes

Locus-specific or unique probes are cosmid (with insert of about 40 kb) and BAC, PAC, or P1 (with inserts of about 125 kb) genomic clones and are used for gene mapping, deletion, and amplification analysis, as well as analysis of chromosome rearrangements such as duplication and translocation. These probes have wide application in genetic diagnosis such as identification of microdeletions, deletion of tumor suppressor genes *(10)*, amplification of oncogenes *(11,12)*, and the demonstration of fusion genes involved in the various translocations in cancer *(13–15)*.

1.1.3. Whole-Chromosome Paints

Whole-chromosome painting probes are polymerase chain reaction (PCR)-generated from flow-sorted genomic DNA libraries that are used to identify individual chromosomes or chromosome segments *(16,17)*. Chromosome painting probes can also be generated from PCR-amplified DNA of microdissected chromosome segments or markers of unknown origin *(18,19)*. These chromosome-specific probes are either labeled with a single fluorochrome or with specific combinations of two or more fluorochromes to generate increasing numbers of discernable targets beyond the number of fluorochromes that are available. Using combinatorial labeling with 5 different fluorochromes, up to 31 different targets can be distinguished *(20,21)*.

1.1.4. Telomere Probes

Cytogenetic analysis of chromosome ends is often challenging, as most of the chromosome ends are G-band negative and, thus, lightly stained. Cryptic rearrangements involving these regions are often difficult to detect by routine cytogenetic banding methods. Submicroscopic deletions or rearrangements of the telomeres have deleterious consequences leading to genomic instability and cancer. FISH probes specific to unique telomere sequences are now available commercially, which enable the identification of individual telomeres and their rearrangements. Also, peptide nucleic acid probes can be artificially generated and used instead of natural nucleic acid probes *(22)*.

1.2. Applications of FISH

1.2.1. Multicolor FISH of Metaphase Preparations (M-FISH, SKY, COBRA-FISH, Rx-FISH, mBAND-FISH)

Conventional cytogenetic analysis provides essential information of diagnostic and prognostic importance in patients with genetic abnormalities. However, it has major limitations with respect to the detection of subtle or cryptic chromosomal aberrations and the analysis of highly rearranged chromosomes,

particularly in poorly spread and/or contracted metaphase preparations. Complex chromosomal rearrangements (CCRs) involving whole chromosomes or parts of chromosomes are common in cancer cells, where it is often impossible to identify the chromosomes or chromosome bands that are involved in the rearrangement. New molecular cytogenetic approaches such as multicolor FISH (M-FISH), spectral karyotyping (SKY), combined binary ratio labeling FISH (COBRA-FISH), and multicolor banding (m-BAND FISH) *(21,23–27)* enable improved characterization of these CCRs.

1.2.1.1. Spectral Karyotyping

Spectral karyotyping is a multicolor FISH technique that allows one to simultaneously visualize all human chromosomes represented in different colors by the computer. This facilitates karyotype analysis, particularly for the identification of ambiguous and hidden chromosomal abnormalities. Applications of SKY to screening genomes for chromosomal aberrations in human disease and cancer are numerous. It is useful in mapping chromosomal break points, detecting subtle translocations, identifying marker chromosomes, homogeneously stained regions, and double minutes, and characterizing CCRs *(21,24)*. Although SKY enables the identification of individual chromosomes and small chromosome segments, it is usually limited to the analysis of chromosome translocations without further differentiation of chromosomal subregion. Subregional human genomic probes "painting" several colored chromosome bands have been established by microdissection *(19)* and "reverse painting" *(18)*. However, these probes cannot identify intrachromosomal rearrangements such as inversions. Subchromosomal paints for human chromosomes derived by reverse painting of DNA from primates such as gibbons, which have very close homology, are found to be useful in delineating intrachromosomal rearrangements *(28)*.

1.2.1.2. Comparative Genomic Hybridization

Comparative genomic hybridization (CGH), yet another specialized method of FISH application, is widely used in cancer research and diagnosis *(12,29–31)*. CGH utilizes the hybridization of differentially labeled tumor and reference DNA to normal metaphase chromosomes to generate a map of DNA copy number changes in tumor genomes in a single hybridization experiment. It has been used to complement immunohistochemical, DNA content measurement, and histomorphology to establish a phenotype/genotype correlation in solid tumor progression *(32–37)*. CGH has now become a routine approach and is widely accepted in cancer research. It is the method of choice for obtaining an overview of genetic alterations in cancer cells, especially in solid tumors, where highly abnormal chromosomes and complex karyotypes are common,

but good metaphase preparations are difficult to obtain *(37,38)*. CGH can also be applied to archival paraffin material for retrospective studies *(39–41)*, and because of the ability to amplify and subsequently label small amounts of DNA by degenerate oligonucleotide primer PCR (DOP-PCR) *(42)*, microdissected or laser capture microdissected material can also be used *(43–46)*.

1.2.1.3. Telomere and Telomere Length Measurement (tel-FISH, Q-FISH, Flow-FISH)

Cryptic chromosome rearrangements that are otherwise sometimes missed by routine cytogenetic analysis can be easily identified by chromosome-specific telomeric probes (tel-FISH), which are available commercially. Telomeres containing noncoding DNA repeats at the end of the chromosomes are essential for chromosomal stability and are implicated in regulating the replication and senescence of cells. The gradual loss of telomere repeats in cells has been linked to aging and tumor development. The most important indicator of correct telomere function is telomere length maintenance within the range typical for each species. Quantitative fluorescence *in situ* hybridization (Q-FISH) provides an estimate of telomere length in each individual chromosome with a resolution of 200 basepairs *(47)*. A variation of Q-FISH is the flow-FISH technique, in which a fluorescein isothiocyanate (FITC)-labeled telomere-specific peptide nucleic acid probe is hybridized in a quantitative way to telomere repeats, followed by telomere fluorescence measurements on individual cells by flow cytometry *(48,49)*. It offers the advantage of looking at telomere fluorescence in different subpopulations of cells.

1.2.2. Interphase FISH

One of the theoretical advantages of FISH over conventional cytogenetics is the ability to identify small genetic alterations in both dividing and nondividing cells. Interphase FISH has opened up a wide range of applications for clinical diagnosis. It can be used to define chromosomal numerical abnormalities, chromosome rearrangements involving amplification, deletion or translocation of specific chromosome regions, identification of marker chromosomes *(50)*, and copy number of individual genes.

Interphase FISH is of special interest in cancer diagnosis. Cancer tissues, particularly solid tumors, frequently fail to grow and divide in tissue culture and often have a very poor mitotic index and poor-quality metaphase chromosomes for cytogenetic analysis. Interphase FISH allows cytogenetic diagnosis from these specimens that cannot be used in conventional dye-banding analyses. Furthermore, translocation break-point analysis *(51)* and gene copy number analysis of cancer specimens *(52)*, using single-gene probes, have proven to be useful predictors of treatment response and this area of molecular diagnosis is rapidly growing.

Unlike conventional blood or other specimens submitted for conventional genetic analysis, cancer specimens are usually contaminated with large numbers of normal cells which could lead to false-negative results in a test evaluating the presence of a particular cancer abnormality.

1.2.2.1. FISH of Paraffin-Embedded Tissues

Fluorescence *in situ* hybridization of paraffin sections has been described *(53)*, and commercial kits are currently available to perform such analyses. If the FISH target is highly amplified, one may be able to directly identify amplification within the cancer cells of paraffin sections. Background noise from autofluorescence in the tissue section, difficulty assessing an appropriate tissue plane in a high-power view of a thick section, or poor probe penetration, however, could seriously compromise interpretation of low-level amplification, translocation, or deletion *(54,55)*. However, improved techniques for analysis of such fixed tissues by FISH have been reported *(55)*.

High-throughput genetic profiling of tumor tissues by FISH on tissue microarray is a very promising improvement to conventional paraffin section FISH *(56–58)*, where a large number of tissue sections can be labeled and analyzed from one slide (*see* **Fig. 2**; Color Plate 12 following p. 274). This is very economical, time saving and very useful for retrospective studies of prognostic or predictive markers. Wholesale purification of nuclei from paraffin sections could decrease the effects of tissue autofluorescence, probe penetration, or specimen thickness on FISH signal visualization *(59)*; however, the *in situ* verification of the source of the cells is lost when the tissue is destroyed during nuclear isolation procedures. Laser capture microdissection (LCM), a new technique to purify individual or groups of cells from paraffin-embedded tissue sections *(60)*, was used to prepare nuclei from breast carcinoma cells for both FISH and flow cytometric analysis *(61)*. This technique, LCM-FISH, allows identification of normal interphase copy numbers, permitting detection of low-level amplification or potentially single-copy deletions, which would be very difficult in paraffin section FISH (*see* **Fig. 3**; Color Plate 13, following p. 274). LCM-FISH was also very useful in evaluating deletion as a mechanism for loss of heterozygosity (LOH) by allowing comparison between microsatellite analysis and interphase FISH in microdissected breast cancer cells *(62)*. In our experience, purification of nuclei from either whole or microdissected tissue results in much less background staining, likely because most of the cellular RNA is removed from the specimen.

1.2.2.2. Interphase FISH and Prenatal Diagnosis

Interphase FISH also has considerable application to prenatal diagnosis. Prenatal assessments of common aneuploidy can be determined rapidly by interphase FISH, using uncultured amniocyte cells. Noninvasive prenatal diagnosis is also becoming possible with the analysis of fetal cells in maternal circulation.

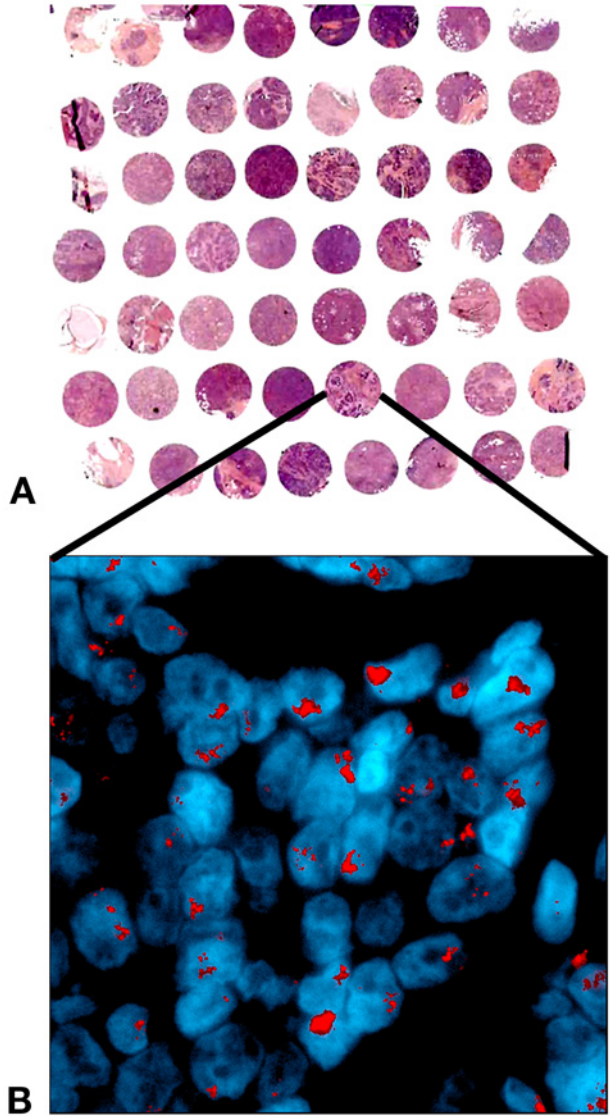

Fig. 2. FISH of tissue microarray breast carcinoma specimens. A tissue microarray block was prepared from 1.5-mm plugs of paraffin-embedded, formalin-fixed human breast cancer specimens. A 6-µm section was cut, deparaffinized, and subjected to FISH with a human erbB-2 genomic BAC probe (red). A hematoxylin/eosin figure **(A)** shows the location of the higher-magnification FISH image **(B)**, which illustrates amplification of the *erbB-2* gene. (*See* Color Plate 12, following p. 274.)

FISH allows rapid detection of specific chromosome abnormalities in uncultured amniotic fluid cells within 2 d of amniocentesis. The technique is typically used in pregnancies at high genetic risk for which a quick result is important in future

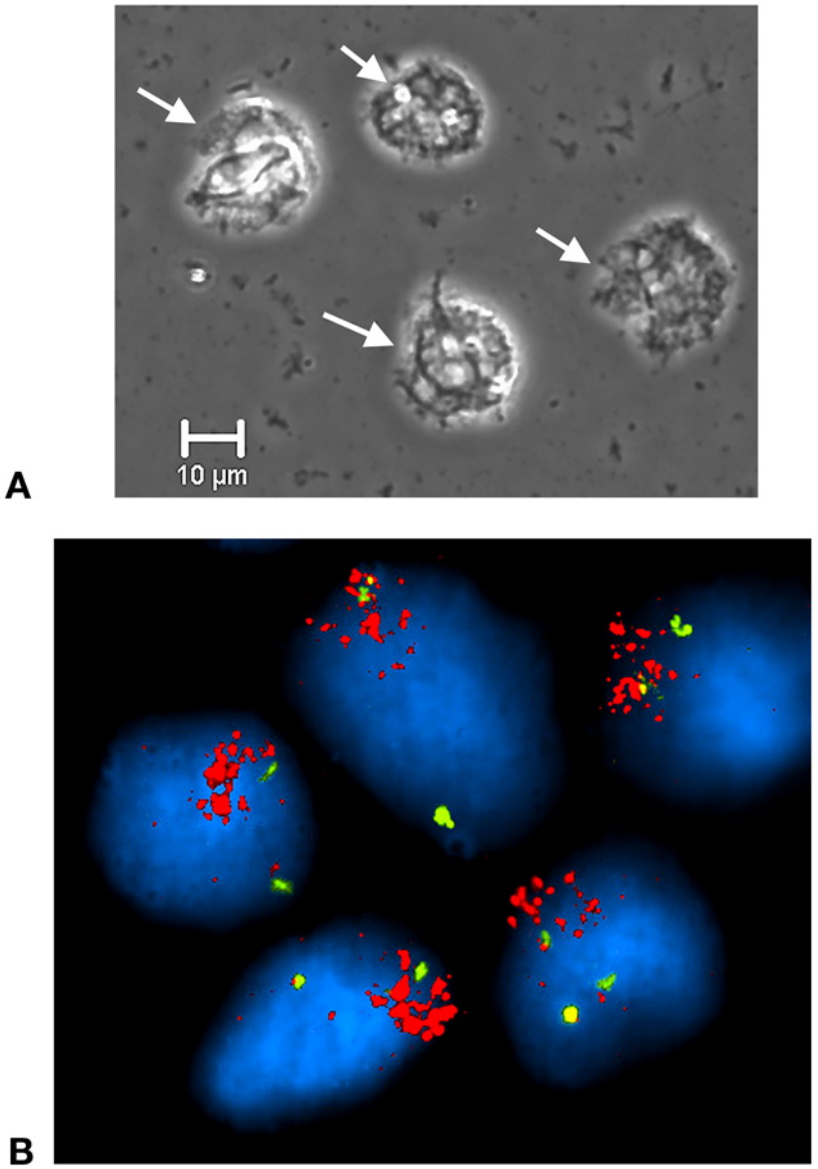

Fig. 3. FISH of LCM-prepared nuclei. Cells were harvested from 30-µm sections of hematoxylin-stained human breast carcinoma by LCM. The nuclei were purified using organic solvents, rehydration, and proteinase K digestion, as described previously ***(61)***, dropped onto clean glass slides, and fixed. The specimens were photographed under phase contrast with the "naked nuclei" indicated by arrows **(A)**. The scale bar indicates 10 µm. The cells were subjected to hybridization with a genomic BAC probe for human erbB-2 (red) and a human centromeric probe for chromosome 17 (green) and show amplification of erbB-2, as in Fig. 2. (*See* Color Plate 13, following p. 274.)

decision making *(63–66)*. Interphase FISH is also a very useful follow-up tool on investigations of uncultured amniotic fluid (AF) cells after finding an uncertain chromosome aberration in a first chorionvilli (CV sample) or AF sample *(67–69)*.

1.2.3. Fiber FISH

Direct visualization of fluorescent probes on extended DNA has been used in high-resolution gene mapping and identification of chromosome break points *(70)*. Recent progress in enhancing the resolution and sensitivity of the FISH technique has further facilitated the physical map construction *(71–73)* of the human genome. For example, fiber-FISH has been used to prepare contigs of the human Y chromosome covering areas that were resistant to easy physical mapping *(74)*. This technique is now only rarely used.

1.3. Microarray-Based CGH

Utilizing the recent advancement of expression microarray technology, microarray-based CGH provides a means to quantitatively measure DNA copy number aberrations and to map them directly onto genomic sequences where genomic DNA is hybridized onto arrayed genomic clones *(75,76)*. Ligation-mediated PCR is an improved method of generating homogenous and highly reproducible amplified DNA from a single cell, with fragment size of 0.2–2 kb *(77,78)*. It has been demonstrated by these authors that arrays generated from ligation-mediated PCR of BAC genomic DNAs provided precise measurements in cell lines and clinical material so that high-level amplification and single-copy alterations could be reliably detected in diploid, polyploid, and heterogeneous backgrounds.

An alternate technique, namely hybridization onto cDNA microarrays, offers significant advantages, most importantly the ability to identify amplified genes rather than amplified genomic regions and to perform expression studies on the same arrays using standard expression microarray approaches *(79–81)*. Thousands of mapped cDNAs are readily available, which facilitate amplicon mapping and identification of new cancer genes (http://www.bcgsc.bc.ca/; http://www.nhgri.nih.gov/; http://www.ncbi.nlm.nih.gov/; http://www.cephb.fr/bio/ ceph-genethon-map.html). A powerful approach to defining putative amplification target genes might combine CGH with results from cDNA microarray analysis, followed by a quick survey of large numbers of uncultured tumors with tissue microarray technology to study the clinical significance of such newly discovered gene amplifications. Array-based CGH, in which fluorescence ratios at arrayed DNA elements provide a locus-by-locus measure of DNA copy number variation, represents another means of achieving increased mapping resolution. Whole genomes of organisms can be prepared using bacterial artificial chromosomes and arrayed onto glass for CGH with 1 Mb resolution, including human *(82)* and mouse *(83)*. It is hypothesized that matrix-based

DNA or RNA hybridization techniques could potentially replace the need for chromosome preparation *(58,75,79–81)*. Although CGH has the advantage of requiring only genomic DNA and has proven to be an important initial screening test for chromosomal gains and losses in tumor progression, array CGH cannot at present detect structural alterations such as isochromosome formation, double minutes, homogenously stained regions, clonal heterogeneity, and so forth.

2. Materials

A number of labeled FISH probes are available commercially (e.g., Vysis, Cytocell, etc.) that are more often used for clinical diagnostic purposes. They are used directly for hybridization as per the manufacturer's instructions. Here, detail labeling and detection protocols for FISH, much of which was previously described by Trask *(84)* and have been subsequently modified *(85)*.

2.1. Reagents

1. Nick translation labeling kit (Gibco, cat. no.18160-010).
2. For indirect labeling: Digoxygenin-11-dUTP, Fluorescein-12-dUTP or Biotin-16-dUTP (Roche, cat. nos. 1-093-088, 1-427-857, and 1-093-070, respectively).
3. For direct labeling: Cy3-dCTP, Cy5-dCTP, or FluorX-dCTP (Amersham, cat. nos. PA53021, PA55021, and PA58021, respectively).
4. DNAse I (Gibco, cat. nos.18047-019).
5. Microspin G50 column (Pharmacia, cat. no. 27-5330).
6. Human DNA: human Cot-1, 250 µL (1 mg/mL; Gibco, cat. no. 15279-011) + 50 µL.
7. Human placental DNA (10 mg/mL; Sigma, cat. no. D4642); Mix and ethanol precipitate with 30 µL 3*M* sodium acetate, pH 5.2. Wash with 70% ethanol and resuspend the pellet in 50 µL TE (*see* **Subheading 2.2., item 9**).
8. Herring sperm DNA (10 mg/mL) (Gibco, cat. no. 15634-017).
9. Sheep anti-digoxygenin Fab fragments: 1/100 dilution (Roche, cat. no. 1214 667).
10. Cy3-conjugated donkey anti-sheep IgG (Jackson ImmunoResearch, cat. no. 713-165-147).
11. Antifade (Molecular Probes, cat. no. S7461).
12. Proteinase K buffer: 50 m*M* Tris-HCl, 10 m*M* NaCl, 10 m*M* EDTA, pH 8.0.
13. Proteinase K (Invitrogen, cat. no. 25530-031).
14. 4′-6-Diamidino-2-phenyindole dilactate (DAPI) stain 1 mg/mL (Sigma, cat. no. D9564).
15. Tissue culture media: modified Eagle's medium (MEM) and RPMI-1640 (Gibco, cat. nos. 11090-081 and 18875-093, respectively).
16. Fetal bovine serum (FBS) (Gibco, cat. no.10437-028).
17. Antibiotic/antimycotic 100X: 10,000 U penicillin/10,000 µg streptomycin/25 µg/mL fungizone (Gibco, cat. no. 15240-062).
18. HEPES buffer, 1 *M* (Gibco, cat. no. 15630-080).
19. L-Glutamine: 200 m*M* (100X) (Gibco, cat. no. 25030-081).

20. Phytohemagglutinin (PHA-M form): Reconstituted in 10 mL sterile water (Gibco, cat. no. 10576-015).
21. KaryoMax™ Colcemid solution: 10 µg/mL (Gibco, cat. no. 15210-016).
22. Actinomycin D: 250 µg/mL in PBS (Sigma, cat. no. A1410).
23. RNAse A (Sigma, cat. no. P8038): Prepare 20 mg/mL in TE and heat-inactivate in a boiling water bath for 20 min to remove DNAse activity.
24. Drierite (BDH, cat. no. B26998).

2.2. Solutions

1. Hybridization mix: 2 mL of 50% dextran sulfate, 1 mL of 20X SSC (pH 7.0), and 5 mL good quality formamide; pH with 1 *M* HCl to 7.00.
2. Denaturing solution: 70 mL formamide, 10 mL 20X SSC (*see* **item 8**), and 20 mL high-quality distilled water (pH to 7.0).
3. Formamide wash solution: 50 mL formamide, 10 mL 20X SSC, and 40 mL high-quality distilled water (pH solution to 7.0).
4. PBS (phosphate-buffered saline) 10X: 80 g NaCl, 2 g KCl, 11.5 g $Na_2HPO_4 \cdot 7H_2O$, 2 g KH_2PO_4. Make up to 1 L with distilled water and autoclave.
5. Stop buffer: 0.5 *M* EDTA (pH 8.0).
6. McIlvaine's buffer pH 7.0: 0.63 g citric acid (anhydrous) and 6.19 g sodium dibasic phosphate (anhydrous) in 500 mL water.
7. Counterstain: 2 µL DAPI stain (1 mg/mL stock) (Sigma, cat. no. D9564) in 50 mL McIlvaine's buffer.
8. 20X SSC: 175 g NaCl and 88 g Na-citrate (anhydrous) in 1 L water.
9. Tris-EDTA (TE): 10 m*M* Tris-HCl, 1 m*M* EDTA.
10. Antibody blocking solution: 0.1 *M* sodium phosphate buffer, pH 8.0, 0.1% NP-40 detergent, 0.02% sodium azide, 5% nonfat dried milk. Mix and let it stand several days at 4°C. Centrifuge for 15 min at 200*g* to remove insoluble milk proteins. Store at 4°C.
11. Antibody wash solution: 2X SSC, pH 7.00, and 0.005% 3[(cholamidipropyl) dimethyl ammonio]-propane sulfonate (CHAPS) detergent (Calbiochem, cat. no. 220 201).
12. Trypsin-EDTA: 0.25% trypsin and 1 m*M* EDTA (Gibco, cat. no. 25200-056).
13. Cytogenetics fixative: 1 : 3 glacial acetic acid and anhydrous methanol.
14. Hypotonic solution: 0.075 *M* KCl solution, pH 7.0.
15. Carlsberg solution: 0.1% Sigma protease XXIV, 0.1 *M* Tris-HCl, 0.07 *M* NaCl, pH 7.2.

3. Methods

In this section, we discuss the actual methodology for specimen preparation and the performance of FISH for the more common applications that can be performed in the average laboratory or institution. We will discuss metaphase preparations from synchronized fibroblasts and lymphocytes and interphase preparations from tissue specimens. We will then discuss basic probe labeling, hybridization of the labeled probe to denatured genomic DNA in the specimen, detection of the labeled probe, and visualization of the specimen showing *in situ*

hybridization. Finally, there will be a discussion of specific applications for performing FISH on nuclei isolated from formalin-fixed, paraffin-embedded whole-tissue sections, performing FISH on formalin-fixed, paraffin-embedded tissue sections or tissue microarrays, and, finally, FISH on nuclei from LCM specimens.

3.1 Specimen Preparation

3.1.1. Metaphase Preparation From Fibroblast Culture

1. Two to three days prior to performing the chromosome spreads, split the fibroblast cells 1 : 3 into new flasks with 10 mL MEM culture media and HEPES buffer, 20% FBS, and 1% penicillin/streptomycin and allow the cells to grow to 50–60% confluency. Feed the cells with fresh media 12–14 h prior to harvesting.
2. Add Colcemid to a final concentration of 0.02 µg/mL for 3–4 h.
3. Transfer the media and add 0.5 mL of trypsin-EDTA to detach the cells (1–2 min). Collect the cells in a tube and centrifuge at 400*g* for 10 min. To the gently vortex-resuspended pellet, add 10 mL of warm (37°C) freshly made KCl solution (0.075 *M*) and incubate at 37°C for 30 min.
4. Add 4–5 drops of cold fresh Cytogenetics fixative to the cells in KCl (*see* **Note 1**) and centrifuge at 400*g* for 10 min. To the gently vortex-resuspended pellet, add 10 mL of cold fixative dropwise and with gentle vortexing (4°C) and centrifuge again for 10 min. Repeat this twice. The pellet must be fully resuspended prior to adding the fixative in order to minimize clumps and maximize yield.
5. Resuspend the final pellet in a smaller volume of fixative (0.5–1 mL depending on the size of the pellet). Drop 1–2 drops of this final suspension onto a clean slide and immediately place it on a warm plate (at 37°C) to dry. Warm the slides for 4 h and let them age at room temperature for 3–4 d (*see* **Note 2**). Better spreading might require dipping the slides into cold water and dropping the suspension onto the wet slides and/or from greater height (0.3–1.0 m). Where available, a thermotron (Cytogenetic drying chamber) can be used, which provides an optimum and controlled temperature and humidity environment for achieving ideal spreading results.
6. Seal the slide box in a Ziploc™ plastic bag with silica gel or Drierite and store them at –20°C until further use (*see* **Note 3**).

3.1.2. Metaphase Preparation From Lymphocyte Culture

1. For metaphase preparations from lymphocytes, add 0.5 mL of whole blood in 10 mL of RPMI 1640 tissue culture media with 20% fetal calf serum, 1% penicillin/streptomycin, 1% L-glutamine, 20 U of heparin, 20 U of HEPES buffer, and 4% PHA. Incubate at 37°C for 72 h.
2. Add Colcemid to 1 µg/mL final concentration at the 68th h of culture. Incubate at 37°C for 4–5 h.
3. Centrifuge at 400*g* for 10 min. To the gently vortex-resuspended pellet, add 10 mL of prewarmed 0.075 *M* KCl and incubate at 37°C for 30 min.
4. Continue as in **step 4** of **Subheading 3.1.1.**

3.1.3. Interphase Nuclei Preparation From Fresh or Frozen Tissue Samples

1. With a sterile scalpel, cut a small piece (0.1–0.2 cm) of fresh or frozen tissue and gently touch it onto four to five spots onto a clean slide (one might want to score a circle on the underside of the slide with a diamond pencil to mark the spot for hybridization and visualization purposes).
2. Semidry (*see* **Note 4**) and fix the cells in fresh Cytogenetics fixative for 10 min. Air-dry and then heat on a warm plate at 37°C for 4 h. Age the slides for 3 to 4 d at room temperature and store them until used further for FISH.

3.2. Probe Labeling, Hybridization, and Visualization

Probes can be labeled either by direct or indirect labeling methods. In direct labeling, the detectable molecule (Cy3-dCTP or Cy5-dCTP) is bound directly to the nucleic acid probe so that after hybridization and posthybridization washing, it can be visualized immediately. This type of labeling gives less background, but the signal is often weak. In indirect labeling, the probe is first labeled enzymatically with a hapten (digoxygenin or biotin, **Subheading 3.2.1.**, **step 1**), which in the second step is accessible to the respective antibody (antidigoxygenin or avidin, **Subheading 3.2.2.3.**, **step 3**) to which the fluorescent molecule is tagged. This type of labeling gives a brighter signal because of the attachment of a large number of antibody molecules to the hapten, which leads to an amplification of the fluorescent signal.

3.2.1. Probe Labeling

Perform the nick translation labeling method using a Gibco Nick Translation Kit (*see* **Note 5**). The following protocol is as previously published with some modifications *(85)*:

1. In a microfuge tube, add the following:

   500 ng probe DNA and dH_2O to a total volume of 18.5 µL;
   2.5 µL of dTTP minus dNTP mix for indirect labeling or dCTP minus dNTP mix for direct labeling;
   1 µL Digoxygenin-11-dUTP for indirect labeling or 2.5 µL Cy3-dCTP for direct labeling.
2. In a separate tube, add 1 µL DNAse I to 20 mL of dH_2O (*see* **Note 6**). Add 1 µL of this to the above mixture. Mix well and add 2.5 µL Pol I/DNAse I mix (0.5 U/µL DNA Polymerase I, 0.4 mU/µL DNAse I). Incubate at 16°C for 90 min.
3. Add 7 µL of FISH stop buffer (0.5 *M* EDTA, pH 8.0) and 25 µL TE. Desalt through a Microspin™ G50 column as per the manufacturer's instructions.
4. Dry the sample on a Speedvac™ and resuspend the sample in 20 µL TE.

   Labeled probes can be stored at –20°C in the dark for up to 1 yr.

3.2.2. Hybridization

3.2.2.1. Hybridization Mix and Probe Denaturation

3 µL of labeled probe (from the 20-µL labeled probe of **Subheading 3.2.1.**);
1 µL of human DNA (Human Cot + Placental DNA, final concentration per 20-µL reaction: 5 µg and 10 µg, respectively);
1 µL Herring sperm DNA (final concentration per 20-µL reaction: 10 µg);
15 µL Hybridization mix.

Mix well and denature at 80°C for 8 min (*see* **Note 7**). Incubate at 37°C for 15–30 min prior to application to the slide, to suppress repetitive DNA-associated background.

3.2.2.2. Denaturation of Target DNA

Incubate aged slides (at least 3–4 d old) at 80°C for 4 min in 70% formamide solution (pH 7.0). Immediately quench in ice-cold 70% ethanol and dehydrate through 80%, 90%, and 100% ethanol and dry the slides on a warm plate (37°C) for 5 min.

Add the hybridization mix with the probe from **Subheading 3.2.2.1.** onto the prewarmed, denatured slide and cover-slip it. Seal the cover slip with rubber cement. Incubate the slide in a humidified chamber at 37°C overnight (chamber can be made by wetting paper towels with water and putting the slides on a pipet-tip rack onto the towels within a sealed Tupperware™ or like container).

3.2.2.3. Visualization

3.2.2.3.1. Primary Antibody Reaction for Indirectly Labeled Slides

1. After overnight hybridization at 37°C, rinse in PBS to remove excess probe. Wash the slides with 50% formamide wash solution, three times for 5 min each, at 42°C and three times for 5 min each with 2X SSC at 42°C.
2. Add 100 µL antibody blocking solution, cover slip the slide, and incubate at 37°C for 30 min in a humidified chamber.
3. Make primary antibody solution by adding 1 µL sheep antidigoxygenin (1:100 dilution) to 99 µL antibody blocking solution. Centrifuge at 10,000*g* for 5 min. Add the supernatant to the slide after blocking for 30 min, cover slip, and incubate at room temperature for 1 h.
4. Rinse slide with PBS and wash three times with antibody wash solution for 5 min each. Again, block with blocking solution for 5 min. In a separate tube, add 1 µL Cy3-conjugated anti-sheep IgG (1:50 dilution) to 99 µL blocking solution and add this to the slide after centrifugation, as earlier, cover slip, and incubate for 45 min at room temperature.
5. Rinse in PBS and wash in antibody wash solution three times for 5 min each at room temperature.
6. Rinse in PBS and counterstain.

3.2.2.3.2. Posthybridization Washing for Direct Labeling

For directly labeled slides, after overnight hybridization, proceed as follows:

1. Rinse the slide in PBS and wash in 50% formamide solution at 42°C, three washes of 5 min each.
2. Rinse three times for 5 min each in 2X SSC.
3. Rinse in PBS and counterstain.

3.2.2.3.3. Counterstaining

After the washing steps for both of the labeling procedures, stain the metaphase chromosomes and/or interphase nuclei as follows. In 50 mL McIlvaine buffer, add 2 µL of DAPI stain (1 mg/mL stock). Stain the slides for 4 min for metaphase spreads and 2 min for interphase nuclei. The DAPI might need to be titrated to a much lower concentration (0.1–2 µL DAPI stock per 50 mL of buffer) to decrease very bright interphase nuclei staining.

After staining with DAPI, treat the metaphase preparations with Actinomycin D for 5 min for DA-DAPI banding (not necessary for interphase specimens). Rinse in PBS, air-dry, and mount in glycerol-based antifade. Slides can be stored at 4°C in the dark until visualization, if less than 12 h (*see* **Note 8**), or at –20°C for longer periods (up to several months). Examples of multicolor FISH on metaphase chromosomes are shown in **Fig. 1.**

3.2.3. FISH Analysis of Formalin-Fixed, Paraffin-Embedded Tumor Samples

Fluorescence *in situ* hybridization on a formalin-fixed paraffin-embedded tissue sample is difficult because of poor probe penetration as well as autofluorescence from the fixed tissue. The following protocol ***(55)***, which results in the isolation of whole nuclei, has demonstrated good FISH results in such tissues. For treatment of paraffin sections in which the morphology is to be retained, *see* **Note 9**.

1. Deparaffinize the tissue in xylene for 30 min (at least two changes of fresh xylene).
2. Rehydrate through a 100%, 95%, 70%, and 50% ethanol series and finally wash in dH_2O.
3. Digest the tissue in Carlsberg solution for 1 h at room temperature. Rinse in water.
4. Treat with 100 µg/mL heat inactivated Rnase A (Sigma) in 10 m*M* Tris-HCl, pH 7.5, 1 m*M* EDTA, 0.3% NP-40 for 15 min at room temperature.
5. Remove nuclei (carefully draw supernatant through wide-bore pipet or filter through nylon mesh to remove tissue fragments).
6. Drop one to two drops of the above suspension onto a clean glass slide, air-dry, and fix in Cytogenetics fixative (can be stored at this time). Bake 4 h at 37°C and store as described previously.
7. Allow slides to come to room temperature and remove from storage box.
8. Incubate in 50% glycerol/0.1X SSC at 90°C for 3 min.
9. Cool to room temperature in 2X SSC.

10. Denature for 5 min at 80°C in 70% formamide in 2X SSC and quench in ice-cold 70% ethanol.
11. Dehydrate in ethanol series.
12. Incubate in Proteinase K (8 µg/mL) solution for 7.5 min at 37°C.
13. Dehydrate in ethanol series and air-dry.
14. Hybridize with probe and proceed as in routine FISH protocol for washing and visualizing.

3.2.4. FISH of LCM-Nuclei

For FISH of nuclei for which the cell of origin must be known, isolation of nuclei from whole tissue ***(55)*** is not acceptable. Cells can be microdissected using an Arcturus PixCell instrument; then the capture polymer is dissolved to release the tissue fragment, allowing enzymatic release of nuclei and subsequent FISH. The procedure is basically as described previously ***(86)***.

1. Cut 20- to 30-µm-thick paraffin sections from the blocks of interest. It is best to start with thicker sections to minimize sectioning of the large nuclei common to neoplasms (*see* **Note 9**).
2. Extensively deparaffinize (three changes of fresh xylene over 10–30 min) and stain with hematoxylin (*not* eosin, which leads to a broad-band fluorescent background).
3. Laser capture microdissect the dehydrated slide using the PixCell microdissection instrument (Arcturus Engineering Inc., Mountainview, CA) according to the manufacturer's protocols (www.arctur.com). You will need to increase the pulse energy and pulse time over the usual to allow capture of cells from the thick section (*see* **Note 10**).
4. Extract nuclei from the LCM caps as follows ***(86)***, using care to avoid flames or sparks and an appropriate fume safety cabinet (*see* **Note 11**). Add 100 µL of fresh chloroform into a 0.5-mL microfuge tube and cap with a CapShur LCM cap containing the microdissected specimen. Invert the tube for 10 s and centrifuge at 3000*g* for 30 s to release the tissue from the "capture" polymer of the LCM cap (*see* **Note 12**). Remove the LCM cap and add 200 µL of anhydrous ethyl ether and mix by inversion (necessary to lower density of the solvent, allowing tissue to pellet).
5. Centrifuge and remove the supernatant. Wash the pellet three times with 400 µL of fresh xylene to remove dissolved LCM cap polymers. Wash the pellet with 400 µL of 100%, 95%, and 70% ethanol, and two washes with TE, pH 8.0. Finally, wash the pellet with Proteinase K buffer (50 m*M* Tris-HCl, 10 m*M* NaCl, 10 m*M* EDTA, pH 8.0) and resuspend in the same solution to a final volume of 100 µL. Add Proteinase K (50 µL at 0.015% to a final concentration of 0.005%). Digest for 60–120 min at 37°C with gentle finger-vortexing every 10–20 min.
6. For FISH analysis, dilute the cells with 350 µL of TE and gently remove the liquid into another 0.5-mL microfuge tube with a large-bore pipet tip (0.5–1 mm) to leave the undigested tissue fragments in the tube. Centrifuge for 5 min at 10,000*g,* followed by careful removal of the supernatant. Resuspend the pellet in 50 µL of TE and drop 2–3 drops of this suspension onto a clean slide. Air-dry the slides and

then fix them in fresh Cytogenetics fix for 5 min and air-dry again. Warm at 37°C for 4 h and either store as usual or proceed to hybridization. (*See* **Notes 13** and **14**.)

4. Notes

1. Slow fixation of cells helps to retain a better morphology of the metaphase chromosomes.
2. Aging of slides is important, as it leads to slow and complete fixation of the cells. This helps in retaining good chromosome/nuclei morphology during the succeeding denaturation and staining procedures.
3. Slides stored at –20°C under desiccation can be used for more than 1 yr without any distortion of the metaphases plates or interphase nuclei, or loss of signal. Remember to allow the slides to come to room temperature before unsealing the storage bag, to minimize condensation.
4. Do not dry the tissue completely after making the imprints.
5. Although dogma suggests that nick translation labeling must be used to ensure optimal penetration of the specimen by the labeled probe, our personal experience has not identified significant differences in FISH signal intensity between random primer or nick translation hapten-labeled genomic probes.
6. Dilution of DNAse I should be optimized by digesting and aliquot of probe with various dilutions of DNAse I (1/1000 to 1/20,000) and electrophoresing a small aliquot of the sample in a 1% agarose gel. Optimal DNA digestion will yield a smear between 200 and 600 bp.
7. If aminoallyl-labeled nucleotides are used for labeling, it is important to denature at 65°C instead of 80°C. A higher denaturation temperature might cause loss of fluorescence.
8. Photobleaching seems to be lessened and the fluor signal seems better if the slides are left overnight prior to visualization.
9. If purification of nuclei is not desirable, bypassing **steps 3–7** will result in treatment of the tissue to improve probe penetration and decrease autofluorescence, without removing nuclei (*see* **Fig. 2**).
10. Some experimentation is required to optimize specimen thickness (for better visualization) vs nuclear yield. **Do not** use adhesive or other coated slides to pick up the paraffin sections from the microtome, or you will not be able to remove the microdissected cells from the glass slide.
11. Some experimentation is required to maximize capture, which will be dependent on section thickness and tissue composition. For the PixCell II, laser energy might require 70–80 mW or even higher for 30-µm-thick sections and pulse duration of 7–8 ms.
12. Be very careful using these solvents! **Diethyl ether is highly volatile, flammable, and potentially explosive if not fresh or stored properly**.
13. It is not uncommon for bits of the capture polymer to remain undissolved. Leave them in the tube for the following steps, but when resuspending the cells for harvest, add more TE up to 700 µL, or perform two pipet extractions (e.g., 350 µL each) to maximize the yield of nuclei, and then centrifuge the nuclei slightly longer (6 min instead of 5 min) to pellet them.

14. For LCM, familiarity with the specimen morphology is essential. For the inexperienced user, it might be helpful to have a picture of the tissue section marked up by a pathologist to guide the microdissection. The morphology of dried, 30-µm-thick tissue sections is often terrible. As well, the tissue thickness precludes dissections of single cells or tiny (four to eight) cell groups unless they are well separated from other cells, as the laser energy required to melt the polymer to penetrate a thick section usually expands the beam width beyond 30–40 µm. Make sure that collagen fibrils are not inadvertently captured, as they will cause lifting of adjacent tissue and contaminate the specimen.

    For poor yields, check that the section thickness is thick enough to avoid nuclear slicing, that the capture polymer is dissolving, that the released tissue sections are not floating and thus being removed with solvent supernatants, and that the centrifugation step is fast enough and long enough to ensure pelleting of the nuclei from your specimens. One can drop a sample of the nuclei on a slide and examine it after air-drying under phase-contrast optics ***(86)*** or stain it with methylene blue or hematoxylin to evaluate the quality of the preparation prior to using it for FISH.

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