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Technical Advances

Thick-Section Fluorescence *in Situ* Hybridization on Formalin-Fixed, Paraffin-Embedded Archival Tissue Provides a Histogenetic Profile

Curtis T. Thompson,*† Philip E. LeBoit,† Petra M. Nederlof,* and Joe W. Gray*

From the Division of Molecular Cytometry, Department of Laboratory Medicine and the Division of Dermatopathology,† Departments of Pathology and Dermatology, University of California, San Francisco, California*

Fluorescence* in situ *hybridization has become a major tool for analysis of gene and chromosome copy number in normal and malignant tissue. The technique has been applied widely to fresh tissue and dispersed formalin-fixed, paraffin-embedded archival tissue, but its use on sections of archival tissue has largely been limited to sections <6 μ thick. This does not provide intact, uncut nuclei for accurate analysis of gene or chromosome copy number. We report here a method of hybridization to sections >20 μ thick that overcomes these difficulties. Key developments were the use of DNA probes directly labeled with fluorochromes and optical sectioning using laser-scanning confocal microscopy. (Am J Pathol 1994, 144:237–243)

Ready availability of well-characterized human tumor tissue is important to tumor research programs, especially those based on the use of fluorescence *in situ* hybridization (FISH) for detection and characterization of genetic changes associated with carcinogenesis and cancer progression. Samples of tumors have been archived for decades during traditional pathological analyses as formalin-fixed, paraffin-embedded specimens. Even today, traditional pathological processing of tissue removed during a surgical procedure dictates that most of the tissue be formalin-fixed for pathological analysis, leaving little fresh tissue available for research analysis. In addition, the coordinated effort required to obtain fresh or fresh frozen tumor tissue is substantial and not consistently successful. Thus, substantial effort has been devoted to development of techniques for genetic and phenotypic analysis of archived tissue.

To date, FISH has been successfully applied to analysis of gene and/or chromosome copy number in cell lines,[1] touch preparations from fresh tissue,[2–4] nuclei dispersed nuclei from formalin-fixed, paraffin-embedded archival tissue,[2,5–8] and thin sections (<6 μ) cut from paraffin blocks.[3,9,10] Analysis of isolated nuclei from paraffin sections eliminates the need for fresh tissue and enables quantitative analysis of gene copy number.[11] However, it is generally useful only on selected or advanced tumors where the tissue being dispersed consists mostly of tumor cells. Additionally, loss of histological architecture requires that a cytological judgment be made regarding the character of each cell (eg, whether a nucleus is from a benign or a malignant cell). Analysis of thin (<6 μ thick) sections does retain the tissue organization. However, quantitative analyses of gene copy number are compromised by the fact that most nuclei are not intact (Figure 1). Statistical corrections can be made to partially correct this artifact,[4,12] although similar corrections used in DNA content estimation by image cytometry can produce false evidence of aneuploidy.[13] In addition, aberrant cells present at low frequency may be missed.

We have overcome this problem by developing a method for FISH analysis of thick (>20 μ thick) sections of formalin-fixed, paraffin-embedded tissue.

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Address reprint requests to Dr. Curtis T. Thompson, Division of Molecular Cytometry, Department of Laboratory Medicine, University of California, San Francisco CA 94143–0808.

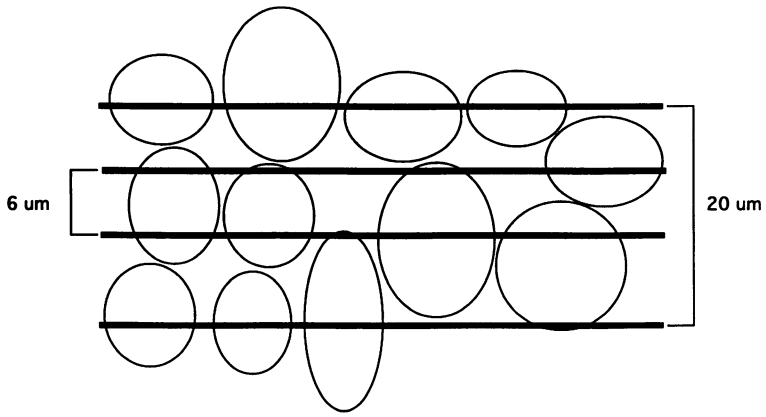

Figure 1. *Proportion of cells left uncut in a 6-μ versus a 20-μ issue section. Almost no nuclei are left uncut in the 6-μ section, while a 20-μ section will contain a layer of cells in the center of the section with uncut nuclei.*

Sections >20 μ thick contain a layer of cells in the center of the section which have not been cut. Our procedure is a modification of that reported earlier by Hopman et al.[3] Changes include steps to maintain the integrity of the tissue section during hybridization, the use of direct-labeled probes to minimize nonspecific background hybridization, and the use of laser-scanning confocal microscopy to reduce fluorescence from out-of-focus regions of the section.

Materials and Methods

Formalin-fixed, paraffin-embedded tissue was obtained from the archives of the Division of Dermatopathology, Department of Pathology, University of California, San Francisco. All tissue had previously been fixed in 10% neutral buffered formalin and processed with standard histological embedding techniques. Tissue was taken from 17 different cutaneous melanocytic tumors, both benign and malignant, all of which were excised and processed from 1990 to 1992. Twenty-micron sections were cut and placed onto slides that had been treated immediately before use with 2-aminopropyltrimethoxysilane (2%, Sigma Chemical Co., St. Louis, MO) in acetone (Baxter Healthcare Corp., McGaw Park, IL). In this process, the slides were incubated for 1 minute in the Silane solution, washed twice for 10 seconds each in acetone, and then washed in distilled water. Alternatively, pretreated slides were purchased commercially (ProbeOn Plus Microscope Slides, Fisher Scientific, Fair Lawn, NJ). The slide-mounted sections were heated at 60 to 70 C on an incline for 1 hour, followed by two washes in xylene (Fisher Scientific) for 10 minutes and 1 hour, respectively. Next, the preparations were heated to 60 to 70 C for 3 minutes to evaporate the xylene, treated in sodium thiocyanate (1 mol/L NaSCN, Sigma) for 10 minutes at 80 C, washed twice in distilled water for 5 minutes each, and heated to 60 to 70 C until dry. The preparations were digested with pepsin (Sigma; 200 mg in 50 ml of 0.2 mol/L HCl) for 5 minutes at 4 C and then for 3 to 7 minutes at 37 C. After digestion, the preparations were placed in 70% formamide (Fisher Scientific) in 2X SSC (300 mmol/L sodium chloride, 30 mmol/L sodium citrate, pH 7.0) at 20 C for at least 3 hours and then at 70 to 75 C for 10 minutes. The preparations were drained and cooled to 37 C.

FISH was performed using an α-satellite, pericentromeric probe specific for chromosome 1 (pUC1.77).[14] Some hybridizations were performed using biotin-labeled probes with secondary detection using fluorescein-avidin and layering with biotinylated anti-avidin as previously described.[15] Most hybridizations were performed using probe DNA labeled directly with fluorescein-12-dUTP (Boehringer Mannheim, Indianapolis, IN) by nick translation using a commercially available kit (GIBCO BRL, Gaithersburg, MD) according to the manufacturer's instructions. The nick translation conditions were optimized to produce probe ranging from 300 to 800 bp. The probe mixture concentration after nick translation was 20 ng/μl. Hybridization with these probes was usually successful despite variations in probe size. Other hybridizations were performed using a probe for the same region directly labeled with Spectrum Green (Imagenetics, Naperville, IL).

For FISH, 30 to 50 μl of hybridization mixture (1 volume of probe mixture, 2 volumes of deionized, double distilled water, and 7 volumes of Master Mix: 1 g dextran sulfate, 5.5 ml formamide, 0.5 ml 20X SSC, and 1.5 ml deionized, double-distilled water, pH 7.0) were heated 70 to 75 C for 5 minutes and applied to each slide preparation at 37 C. After overnight hybridization in a humid environment at 37 C, preparations were washed three times in 50% formamide in 2X SSC, twice in 2X SSC, and once in

0.1X SSC (45 C, 15 minutes). After one wash in PN buffer (100 mmol/L sodium phosphate, 0.05% Nonidet P-40, pH 8.0) at 20 C, the slides were drained and counterstained with 30 μl propidium iodide (PI; 0.1 μg/ml; Sigma) in an embedding medium containing antifade (*p*-phenylenediamine dihydrochloride, 1%, Sigma; 90% glycerol in PBS, pH 7.0).[16] A coverslip was placed over the tissue, and the edges were sealed with a clear nail polish.

Sections were analyzed using a dual detector Olympus laser scanning microscope LSM-GB200 (Olympus Corporation, Lake Success, NY). This confocal system is equipped with a conventional mercury lamp and epi-illumination optics so that samples can be examined visually using conventional optics and optically sectioned using the confocal optics. Thus, tissue preparations were visually scanned using an Olympus 60× 1.4NAA objective. Higher magnification zooming was produced by reducing the size of the scanning field on the specimen to produce magnifications between 75× and 225×. Regions of interest were optically sectioned with the argon ion laser set at a 515-nm emission line, which was made more precise using a 515-nm dichroic mirror. Laser intensity was adjusted to be 1 to 10% of maximum output (25 MW maximum output) using neutral density filters. To avoid analysis of cut nuclei, the focus was set at a midpoint between the upper and lower borders of the tissue. Fluorescence was directed into two separate paths using a beam splitter. Along one path, the light passed through a 515-nm filter and a 520- to 550-nm band pass filter and onto a photomultiplier for detection recorded as a measure of fluorescein (or Spectrum Green) fluorescence. Along the other light path, light passed through 570 nm and 590 nm long pass filters and onto a photomultiplier where it was detected, digitized, and recorded as a measure of PI fluorescence. Optical sections were obtained at 0.2- to 0.4-μ steps under the control of a piezoelectric Z stage. Optical sections were accumulated using a 16-MB RAM IBM PC-AT compatible computer. Images were displayed on the system 14-inch monitor. Optical sections were either viewed singly (20–50 images/nucleus) or summed together to produce a single two-dimensional "projection" image.

At least 30 nuclei for each clinical sample were scored. Closely spaced opposed nuclei that could not be resolved with certainty were ignored. Larger, more intense spots occupying one continuous area were scored as one spot. Small, round nuclei within the melanocyte nests were considered to be lymphocytes and were ignored. In one tissue section of skin, 30 normal keratinocyte nuclei were analyzed to serve as a diploid control.

Results

FISH using the chromosome 1-specific centromeric probe pUC 1.77 to thick tissue sections produced intense signals localized well within the boundaries of PI-stained nuclei. Intact normal keratinocytes showed the expected two hybridization domains (in 30 of 30 normal nuclei evaluated). Tissue samples showed variations in signal intensity, and no hybridization signals were visible on multiple hybridization attempts on several samples. This occurred especially when the embedded tissue was small.

Analysis using optical sectioning microscopy was essential for this analysis, since several nuclei showed hybridization domains in the same x-y plane but differing in location along the z axis and because the thickness of the tissue section produced out-of-focus fluorescence which obscured the signals. Figure 2 shows confocal images taken every 2 μ from the upper to the lower border of a 20-μ section of tissue. Hybridization signals are clearly visible throughout the 20-μ-thick section. The nuclei seen near the edges of tissue (A, B, H, I) show large diameter PI-stained material, indicating that the nuclei at these locations have been physically cut. Only those seen in the central portion (C-G) are entirely contained within the section and thus are suitable for signal analysis. Thus, hybridization signal analysis should be confined to intact nuclei near the center of the tissue section.

Figure 3 demonstrates that histological features of the tissue are apparent in optical sections taken within the thick section. Figure 3A shows a single confocal section of epidermis overlying a nest of malignant melanocytes (arrow). Figure 3B is a volume projection confocal image showing a dermal blood vessel along which malignant melanocytes are singly infiltrating. A single melanocyte (arrow) contains three hybridization signals of a chromosome-1-specific probe (fluorescein puc1. 77). Both figures demonstrate that pathological characterization of cells and tissues is possible in thick sections, even after FISH, so there is no need to compare the thick section being analyzed to separately cut H&E-stained sections. This allows individual cells with distinctive pathological features to be chosen for genetic analysis using FISH.

FISH was performed using two different probe labeling schemes: 1) two-step probe detection in which probes were labeled with biotin or digoxi-

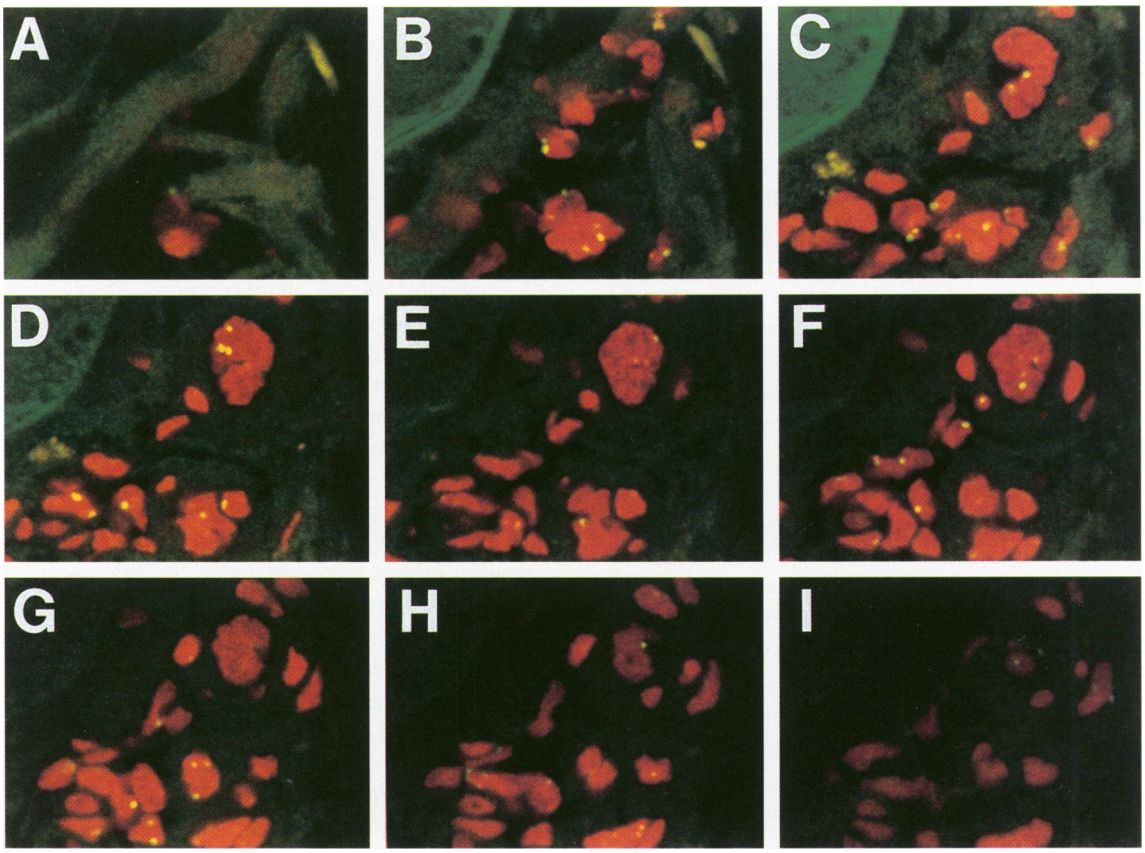

Figure 2. *Confocal section images of dermal tumor cells in cutaneous malignant melanoma hybridized with fluoresceinated chromosome-1-specific DNA probe and counterstained with propidium iodide magnified ×225. The images are samples taken every 2 μ from the upper to the lower border of a 20-μ tissue section and demonstrate that hybridization occurs evenly throughout the thickness of the tissue section. Nuclei near the border of the tissue* (A, B, H, I) *appear as large PI-stained objects, indicating that they have been sectioned along the border of the tissue. Nuclei in the central sections* (C-G) *appear entirely within the tissue section and thus are uncut. These nuclei are suitable for signal analysis.*

genin and detected with fluorescein avidin or fluorescein anti-digoxigenin; and 2) detection with probes directly labeled with a fluorochrome as described above. The background fluorescence produced by two-step probe detection was substantially higher than that generated using directly labeled probes. This could not be reduced even with prolonged washing after staining. In contrast, hybridizations using direct-labeled probe had almost no noise. Analysis of the signals produced using FISH with directly labeled probes showed the signal intensity to be higher for the probes produced by Imagenetics than for those labeled by nick translation. However, both types of probes were employed successfully in this study.

Discussion

We demonstrate in this paper a new method of accurately measuring chromosome copy number using FISH with centromeric probes to intact nuclei in thick sections and correlating this information with pathological information obtained on the same material using confocal microscopy. The ability to perform FISH on sections of formalin-fixed, paraffin-embedded tissue represents an important step forward in cytogenetic research. The advantages of thick section FISH include the wide availability of well characterized tissue with preserved histological architecture in which context cytogenetic information can be obtained (Figure 3) and the opportunity to study lesions early in neoplastic progression, which are often small and therefore inappropriate for analysis by other molecular techniques.

The importance of being able to analyze archived tissue cannot be overemphasized. Such tissue is carefully cataloged during routine pathological analysis and preserved for possible future use. Paraffin blocks are retained as a permanent component of the pathology case and the conclusions of the examining pathologist are recorded in an official legal report which is retained in the medical records.

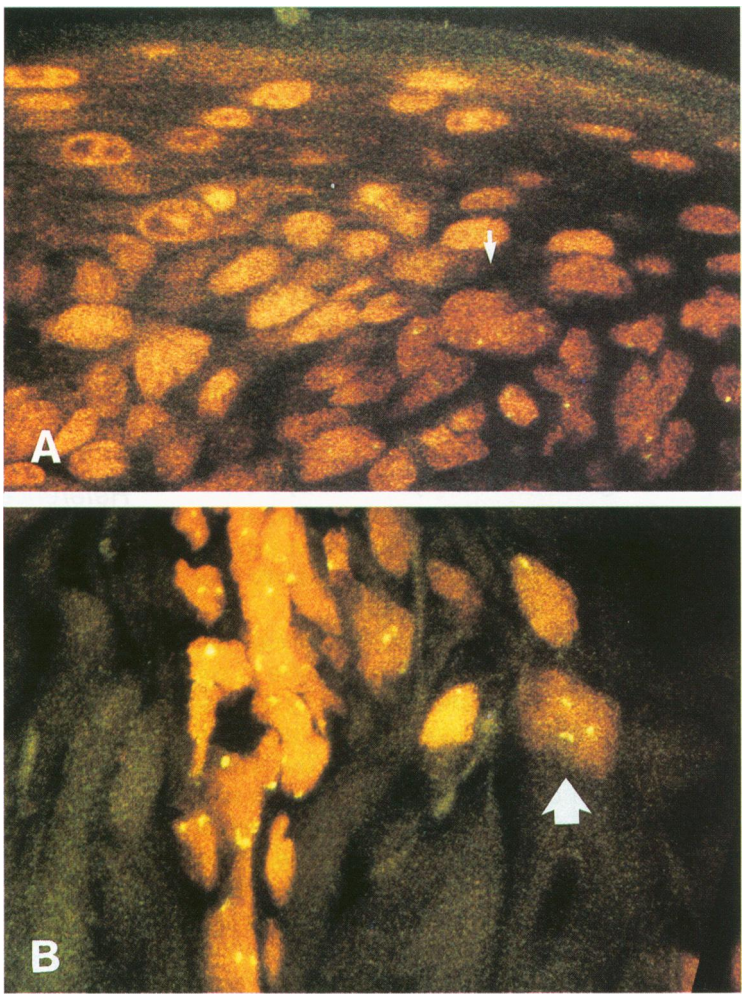

Figure 3. *Confocal imaging of fluorochrome-stained tissue sections (20 μ) of cutaneous malignant melanoma demonstrating that histological and cytological features are easily distinguished with PI-stained tissue. A single confocal image* (**A**) *magnified ×150 ~0.2 μ in thickness of the epidermis with an underlying nest of malignant melanocytes (arrow) shows occasional hybridization signals (fluorescein-labeled puc1.77). Absolute quantitation of signals per cell can only be accomplished through a series of such sections. A series of confocal images projected into a single image* (**B**) *magnified ×225 shows a dermal blood vessel with singly infiltrating malignant melanocytes. A single malignant melanocytic nucleus (arrow) has been entirely sectioned, and the projection reveals three hybridization signals of the chromosome-1-specific probe (fluorescein-puc1.77). Other nuclei in the image may be incompletely sectioned and may not reveal the total number of hybridization signals.*

Clinical studies using archived material are facilitated by the fact that clinical records (including information about treatment, additional biopsies, surgical procedures, and eventual outcome) are retained permanently. Thus, large-scale studies of issues such as the prognostic importance of selected genetic aberrations can be accomplished in an manageable time scale.

The ability to perform molecular cytogenetic analyses on cells within their histological context is important since this allows specific analysis of selected subpopulations within a tumor. For example, one can analyze and compare cytogenetic information from different histological patterns of the same tumor such as invasive versus *in situ* components of a breast carcinoma or malignant melanoma which may exist within a single tissue section. Correlation with traditional pathological tumor grading systems is also possible using this technique. For example, cytogenetic information may be obtained while noting the Gleason score of prostate carcinoma.[17] Many of the pathological tumor grading systems have been correlated well with clinical prognoses, and further correlation with cytogenetic traits may provide links between the cytogenetic aberrations and clinical prognosis.

Analysis of premalignant lesions and early malignant lesions is particularly important to efforts to elucidate the genetic events associated with tumorigenesis and early progression, as the tissue from these small lesions is usually submitted entirely for pathological analysis. Additionally, many early malignancies are not known to be so at the time of the first surgical excision, and diagnosis is only made after pathological analysis. This is especially relevant in research in pigmented lesions where benign nevi and malignant melanoma cannot often be distinguished clinically and must await pathological analysis. Early malignancies often consist of too few cells to be analyzed outside of their histological context.

For example, cutaneous malignant melanoma *in situ* often consists of small nests and single scattered melanocytes within an epidermis composed of many layers of benign cells. Certainly lesions such as these may contain cytogenetic aberrations which are important in the early events of tumorigenesis, and application of this technique to such lesions may provide important cytogenetic information.

Additional work will be needed to develop molecular cytogenetic techniques to the point where they can be applied routinely to analysis of thick sections cut from paraffin-embedded material. Tissue fixation techniques must be studied to determine which conditions produce optimal fixation while retaining the integrity of the DNA. Detection of large, repetitive sequence regions is not usually affected by the fixation conditions; however, detection of unique sequences (eg, using cosmid-sized probes) may require fixation methods which do not markedly alter the DNA.

FISH with nick translated probes to unique sequences in thick sections is not yet reliable in our hands. This approach is currently limited by the relatively low fluorescence intensity that can be generated using directly-labeled DNA probes. This is of no consequence in directly-labeled chromosome-specific, α-satellite probes, since the genomic target and resulting hybridization signal are large. These signals are indeed quite intense. The signal intensity from the unique sequence probes is low at best, and labeling techniques and probe size variations can affect this already low intensity. Perhaps other chemical labeling techniques such as those using PCR amplification with fluorochrome-labeled nucleotides will allow detection of the smaller probes. This development is important, since studies of genetic progression in tumors are likely to focus on coding regions of the genome.

Molecular cytogenetic analyses of thick sections are also limited by the fact that confocal microscopy has not yet been optimized for this purpose. Thus, analysis of signals within nuclei in a tissue section is a slow, laborious process. It is necessary first to find a nucleus or group of nuclei of interest using the eyepiece and then to visualize the nucleus using the confocal imaging system. After determining the upper and lower limits of the nucleus, the nucleus is viewed in multiple sections 0.2 to 0.5 μ from one side to the other. This requires from 10 to 50 separate sections, depending on the size of the cell, and a scanning time of 10 seconds per section, making this process quite long. Because of the usual close association of the nucleus of interest with other nuclei, it is necessary to carefully observe each section to understand the relationship of the other nuclei as they may wrap around each other and the nucleus of interest. Routine application of confocal microscopy will require new software to facilitate this process.

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