Fluorescence *In Situ* Hybridization

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Fluorescence *in situ* hybridization (FISH) is a cytogenetic technique used to detect the presence or absence and location of specific gene sequences. It can visualize specific cytogenetic abnormalities (copy number aberrations) such as chromosomal deletion, amplification, and translocation. FISH has been used in prenatal diagnosis and has served both as a diagnostic and as a prognostic marker for various sarcomas. More recently, FISH entered the field of dermatology in aiding the evaluation of ambiguous melanocytic lesions. This article will discuss the concept of FISH, its application, and its advantages and limitations in dermatology, with an emphasis on melanoma.

WHAT FISH DOES

- FISH is used to visualize specific cytogenetic abnormalities.
- It can serve as a supplementary diagnostic tool in pigmented lesions. However, it should not be used as a stand-alone test.
- FISH cannot replace traditional histopathologic analysis.
- FISH must correlate clinical, pathologic, and molecular information.

LIMITATIONS

- Probe design requires knowledge of specific chromosomal abnormalities to be studied.
- Cutoff signals may differ among laboratories.
- Processing errors, imperfect hybridization, nonspecific binding, photobleaching, interobserver variability, and false-positive and -negative results are possible.

HOW IS FISH PERFORMED?

FISH involves the binding, or annealing, of fluorescence-labeled, target-specific nucleic acid probes to their complementary DNA or RNA sequences and the subsequent visualization of these probes within cells in the tissue of interest. The tissue of interest can either be formalin-fixed, paraffin-embedded sections or fresh-frozen tissue.

First, the DNA or RNA sequences from the tissue of interest are allowed to denature to become single stranded. Next, a FISH probe is selected and applied. The selection of an appropriate FISH probe is a critical step for enhancing its value as a diagnostic test because FISH only detects those chromosomal abnormalities that are specifically targeted by the probes used. Different probes are used depending on the diseases or malignancies under investigation. For example, a large number of recurrent cytogenetic abnormalities have been found in melanomas by comparative genomic hybridization (CGH); these recurrent abnormalities serve as excellent candidates for FISH probes (Song *et al.*, 2011).

Once the probe is selected, the fluorescence labeling of the probe can be done either directly or indirectly. In direct fluorescence labeling, the fluorochrome(s) to be detected by the fluorescence microscope is directly bound to the probe DNA. In indirect labeling, a hapten, which is not visible under a fluorescence microscope, is incorporated into the probe DNA. The hapten is then detected immunohistochemically by a fluorophore-tagged antibody directed against the hapten. Next, the fluorescent-labeled probe and the target DNA or RNA sequences are brought together in the hybridization process, during which the fluorescent-labeled probe anneals to the targeted sequences. Posthybridization washings remove excessive unbound probe. The slides are then examined. Principles of FISH are illustrated in Figure 1.

Because FISH can be performed on formalin-fixed, paraffin-embedded tissue, it is possible for a pathologist to select a specific area or areas of tumors to be examined by FISH. This enables correlation between FISH results and tumor morphology under conventional light microscopy. For more details about FISH, readers are referred to the *Fluorescence In Situ Hybridization (FISH)—Application Guide* (Liehr, 2009).

INTERPRETATION OF FISH

Each fluorescently labeled probe that hybridizes to a cell nucleus in the tissue of interest appears as a distinct fluorescent dot. Each dot identifies a single copy of the chromosomal locus with a homologous DNA sequence. Diploid nuclei will have two dots. If there is duplication in the region of interest,

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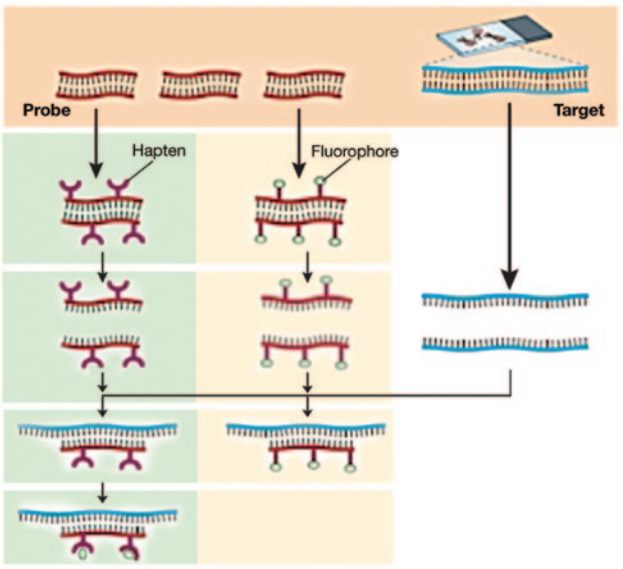

Figure 1. Schematic diagram of the fluorescence *in situ* hybridization (FISH) technique. Reprinted from O'Connor, 2008.

the gain will result in more than two dots. Conversely, if there is a loss in the region of interest, one or no dots will result. In practice, several probes can be combined into a single multicolor FISH experiment. For example, different probes can be labeled with different colors such as red, green, yellow, and aqua, which allows simultaneous interrogation of multiple cytogenetic signatures. Overlapping wavelength spectrums of the currently available fluorochromes limit the maximum number of probes in a single experiment to four (Gerami and Zembowicz, 2011). The number of dots per nucleus with a specific fluorescent color can be detected either manually or with software designed for automated analysis. Because of the intrinsic variability of FISH signals, a sufficient number of cells must be examined to yield a meaningful result.

FISH results are usually reported as a percentage of nuclei containing more than two copies of a particular locus of interest or as a percentage of cells showing a loss or gain of a particular chromosomal region. The cutoff signals, or the exact percentage of cells needed to be considered an abnormal FISH result, must be determined and validated with appropriate controls for each probe. Because the cutoff values are empirically derived, the values may differ among laboratories. Needless to say, having rigorously interrogated cutoff values is important to the robustness of a FISH assay (Gerami and Zembowicz, 2011).

WHAT FISH CANNOT DO: COMPARISON BETWEEN FISH AND CGH

To perform FISH, one must know what one is "FISHing" for. The FISH result is only positive or negative in relation to the interrogated chromosomal region. This is in contrast to CGH, in which copy number aberrations of the entire genome in a tissue of interest are interrogated in a single experiment. Compared with FISH, CGH is more expensive and has a longer turnaround time. Because cells must be microdissected, CGH typically requires paraffin block preparation. In addition, copy number changes must be present in at least 30–50% of the cells for them to be evident on CGH analysis, whereas FISH requires only 20–30 well-visualized cells to provide an accurate count of fluorescence signals. As a result, FISH can be used both in large bulky tumors and in tumors in which the malignant component only contributes to a small proportion of the overall cellular populations. Last, FISH probes can demonstrate balanced translocations that are not detectable at CGH resolution (Gerami and Zembowicz, 2011).

FISHING IN DERMATOLOGY: MELANOMA AND MORE

In contrast to melanoma and a subset of Spitz nevi, melanocytic nevi do not show chromosomal aberrations after karyotyping or CGH. These cytogenetic differences have been exploited to aid in the diagnosis of ambiguous melanocytic lesions. A landmark study to identify the most accurate FISH probes for melanoma was carried out in 2009 (Gerami *et al.*, 2009a). Fourteen candidate cytogenetic abnormalities detected on CGH in prior studies were tested through FISH on 148 melanomas and 153 nevi (including 17 Spitz nevi and 30

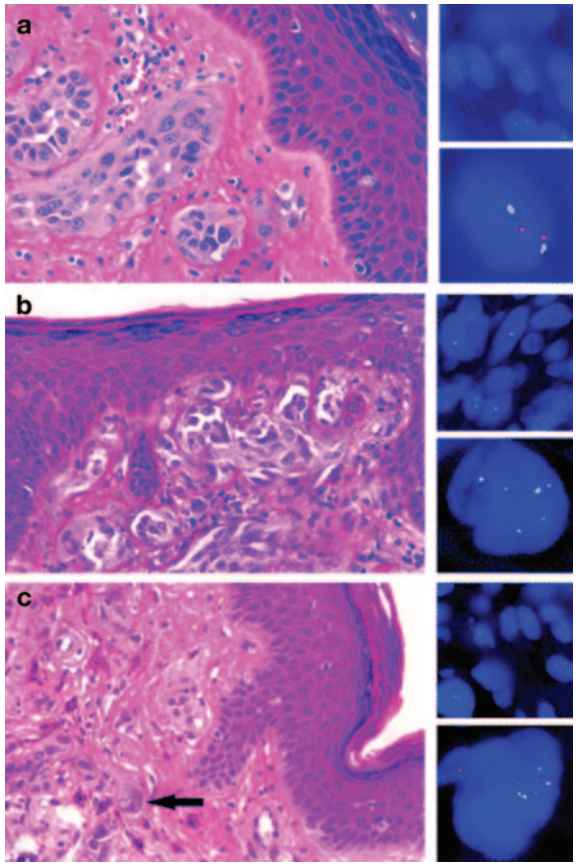

Figure 2. Histology and dual-target fluorescence *in situ* hybridization (FISH) with probe RMC11B022 for chromosome 11p and RMC11P008 for chromosome 11q. (**a**) Case 2, normal CGH measurement; (**b**) case 13, gain of chromosome 11p; and (**c**) case 15, normal CGH measurement. FISH shows that infrequent cells with large nuclei (arrow) are polyploid; green signals, chromosome 11p; red signals, chromosome 11q. Reprinted with permission from Bastian *et al.*, 1999.

dysplastic nevi). A set of four probes targeting 6p25 (RREB1), 6q23 (MYB), 11q13 (CCND1), and centromere 6 (CEP6) were determined to offer the best sensitivity and specificity, 86.7 and 95.4%, respectively. The optimal discriminatory algorithms for defining positive FISH results based on these four probes were also established (Gerami *et al.*, 2009a). These four probes are now commercially available and have been tested in a number of subsequent studies to aid in the diagnosis of ambiguous melanocytic lesions. FISH has been used to distinguish nevoid melanoma from mitotically active nevi (Gerami *et al.*, 2009b).

The majority of Spitz nevi have a normal chromosomal analysis; however, a subset of Spitz nevi have been found to have increased copy number of chromosome 11p (Figure 2) (Bastian *et al.*, 1999). The increase in 11p has not been found in melanoma, allowing for possible differentiation in difficult-to-diagnose spitzoid melanocytic neoplasms.

FISH may play a future role in determining prognosis and identifying tumors with greater metastatic potential, although the clinical utility of these have yet to be determined. For example, FISH analysis for monosomy 3 has helped confirm the diagnosis of metastatic uveal melanoma (Busam *et al.*, 2012).

Although FISH can serve as an adjunct in diagnosis of melanocytic lesions, there are intrinsic limitations to its results such as processing errors, imperfect hybridization, nonspecific binding, photobleaching caused by prolonged light exposure, and interobserver variability, as well as false-negative and false-positive (tetraploidy) results. Furthermore, because melanomas are genetically heterogeneous, different genetic aberrations may be seen in different sections of the same tumor, underscoring the importance of selecting the most appropriate area(s) for FISH analysis.

Use of the FISH technique in other areas of clinical dermatology has not been as well established. For example, although FISH is not routinely used in the diagnosis of cutaneous lymphomas, recent publications investigating specific gene rearrangement, deletion, or translocation have shown the potential for future applications in cutaneous T-cell lymphoma to provide a biologic basis for possible gene-directed therapy as well as prognosis (Marty *et al.*, 2008; Pham-Ledard *et al.*, 2010). Figures 3 and 4 show the use of FISH to identify IFN regulatory factor 4 gene (*IRF4*) translocation in certain cases of cutaneous T-cell lymphoma. Figure 3 shows in schematic fashion how break-apart, dual-color fluorophore-labeled probes can be used to demonstrate translocations, as well as extra copies of the locus of interest. *IRF4* gene probes directed against the 5′ and 3′ ends were differentially labeled (one red, one green) and hybridized. Figure 4 shows a comparison of binding to normal and diseased tissues; the FISH pattern in Figure 4d is normal, with dual color (red and green). In Figure 4h, the signal pattern is split (one locus is dual color, but the other red and green are split), which is consistent with a translocation. Figure 4l demonstrates an extra signal, which is consistent with an extra copy of the *IRF4* locus. More recently, FISH has been utilized to assess clonality in bone marrow and skin infiltrates in patients with neutrophilic dermatoses and myeloid malignancy (Sujobert *et al.*, 2012).

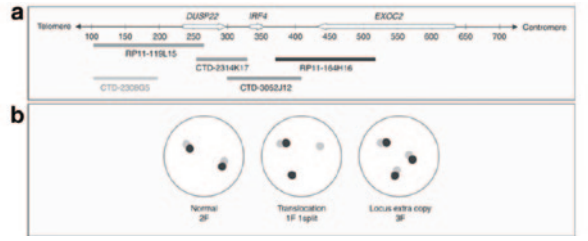

Figure 3. *IRF4* locus-specific fluorescence *in situ* hybridization (FISH) strategy. (**a**) Schematic representation of all BAC clones hybridizing to the 6p25 region used in this study. RP11-119L15, CTD-2317K17, and CTD-3052J12 BAC clones, which showed nonspecific hybridization on chromosome 16, were discarded. The break-apart BAC probe strategy used CTD-2308G5 as the 5′ *IRF4* BAC probe and RP11-164H16 as the 3′ *IRF4* BAC probe. (**b**) The FISH signal pattern expected in interphase nuclei samples. Normal nuclei would exhibit a two-fusion (2F) signal pattern corresponding to the juxtaposition of BAC clones probes. Nuclei with an *IRF4* locus break point, suggesting translocation, would show a split signal pattern (1F–1R–1G). Nuclei with extra copies of a nonrearranged *IRF4* locus should exhibit more than a 2F signal pattern. Reprinted with permission from Pham-Ledard *et al.*, 2010.

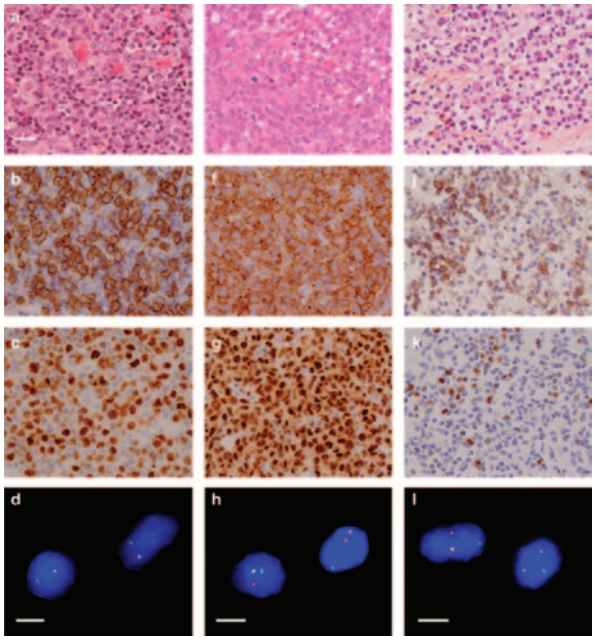

Figure 4. Histological, immunophenotypical, and fluorescence *in situ* hybridization (FISH) aspects of three typical cases with and without *IRF4* locus rearrangement. Left: Lymph node section of cutaneous anaplastic large-cell lymphoma (C-ALCL) without *IRF4* locus rearrangement. (**a**) Hematoxylin–eosin and safran (HES). Bar = 20 μm. (**b**) Positivity of CD30 immunostaining. (**c**) Immunostaining shows expression of multiple myeloma antigen 1 (MUM1) by more than 85% of tumor cells. (**d**) Normal FISH signal pattern (2F). Bar = 5 μm. Middle: Skin section of case 6 with C-ALCL and *IRF4* rearrangement. (**e**) HES, ×400. (f) Positivity of CD30 immunostaining. (**g**) Immunostaining shows MUM1 expression by more than 85% of tumor cells. (**h**) Split FISH signal pattern (1F–1R–1G). Bar = 5 μm. Right: Skin section of case 8 with transformed mycosis fungoides and *IRF4* locus rearrangement. (**i**) HES, ×400. (**j**) Positivity of CD30 immunostaining. (**k**) Immunostaining shows MUM1 expression by 10–50% of large tumor cells. (**l**) FISH signal pattern shows an extra signal of SpectrumGreen-labeled RP11-164H16 (2F+1G extra signal), indicating that the break point maps to the 6p25 region in the genomic area corresponding to the RP11-164H16 sequence. Bar = 5 μm. Reprinted with permission from Pham-Ledard *et al.*, 2010.

QUESTIONS

1. What does FISH detect?

A. Protein structure abnormalities.

B. Specific chromosome copy-number aberrations.

C. Presence of specific antigens.

D. Presence of complement.

2. Where does the FISH probe localize to?

A. Golgi apparatus.

B. Cytoplasm.

C. Cell membrane.

D. Nucleus.

3. What is the FISH probe composed of?

A. Proteins.

B. Lipids.

C. Carbohydrates.

D. Nucleic acids.

4. What is the maximum number of FISH probes that can be used in a single experiment?

A. Two.

B. Three.

C. Four.

D. Five.

Answers to the questions and an opportunity to comment on the article are available on our blog: http://blogs.nature.com/jid_jottings/.

SUMMARY

FISH is a powerful technique used to visualize specific cytogenetic abnormalities. Its most significant role in dermatology to date lies in its ability to aid in the diagnosis and management of ambiguous melanocytic lesions. Although recent studies have suggested that FISH can be used as a supplementary diagnostic tool in pigmented lesions, FISH must not be used as a stand-alone test, and it cannot replace traditional histopathologic analysis. One must correlate clinical, pathologic, and molecular information. "FISHing" in dermatology continues to evolve, and we look forward to future studies to further delineate its roles in various dermatologic diseases.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Answers and a PowerPoint slide presentation appropriate for journal club or other teaching exercises are available at http://dx.doi.org/10.1038/jid.2013.120.

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