FISH Analysis for the Detection of Lymphoma-Associated Chromosomal Abnormalities in Routine Paraffin-Embedded Tissue

TROUBLESHOOTING

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Web site

The authors maintain a companion Web site that can be accessed at the following address**:**
www.tissuemarkers.org.uk/FISH_review
This site contains additional information on reagents and equipment and will be updated at intervals to reflect changing methodologies.

Troubleshooting

We describe below a number of common problems experienced in applying FISH to paraffin-embedded tissue and suggest some possible solutions.

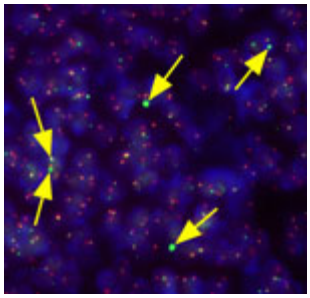
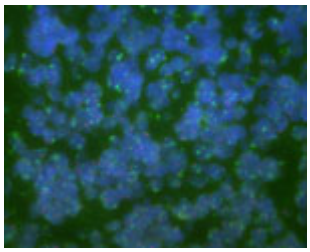
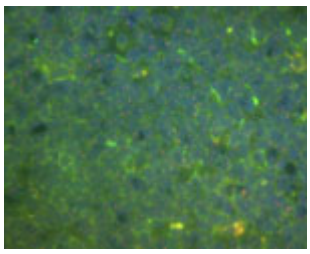
Excessive background fluorescence

Inadequate washing

If the washing time is too short or the temperature too low, background fluorescence may be seen. The temperature of wash solutions should be confirmed with a thermometer before washing slides. If more than 10 slides are to be washed, the solutions should be allowed to reheat to the required temperature before washing.

Naturally occurring background

Several phenomena are apparent that can cause confusion for inexperienced observers when viewing FISH-labeled paraffin sections. Granulocytes tend to fluoresce brightly and should be ignored, and red blood cells will fluoresce brightly in all channels. Single spots of background (resembling FISH signals) may be troublesome, but they can be recognized because they tend to fluoresce in several channels, do not always overlie nuclei and tend to be more sharply defined than true FISH signals. These background spots may also lie in a slightly higher focal plane than true FISH signals.

	**Background spots** MYC break-apart probe applied to Burkitts lymphoma. The yellow arrows indicate green background spots. These spots may be larger than the genuine FISH signals, have very sharply defined edges, fluoresce in all channels and be more intense in colour. Areas with high amounts of background spots should be avoided although with care interpretation of FISH signals is often possible.
	**Follicular lymphoma: green background** Follicular lymphoma analyzed with t(14;18) dual-fusion FISH probe. High levels of green background are seen in this area. Such areas should be avoided or interpreted with care. Narrow band filters specifically designed for the probes being used may reduce such background. Increasing pepsin digestion times and a more stringent post-hybridization washing may also help.
	**High autofluoresence** Strong green autofluoresence is present resulting in the green FISH signals and nuclear borders being difficult to identify. Such samples should be repeated with increased pepsin incubation times.
	**Mantle cell lymphoma: green background** Strong green fluorescence is present, possibly due to inadequate pepsin digestion or poor fixation/preparation of tissue sample. Signals can be interpreted with care but analysis should be repeated or an area identified with lower background.

Larger versions of these images can be viewed by clicking on the images available at
www.tissuemarkers.org.uk/FISH_review/troubleshooting.html

Weak or absent FISH signals

Sub-optimal tissue fixation and embedding

Several factors involved in the preparation of paraffin-embedded tissue have been identified as affecting the preservation of tissue (reviewed by Srinivasan et al, 2002). For instance, the usage of buffered formalin is recommended to obtain good hybridizations. Undoubtedly some samples will have been handled in such a way that good FISH results cannot be obtained. However, it is worth altering the proteolytic or other pre-treatment conditions at least once if initial results are uninterpretable.

Inadequate demasking

In our experience inadequate demasking times/temperatures (pressure cooking or chemical treatment) is a common cause of weak signals. If weak signals are observed the pressure cooking or chemical pre-treatment times should be increased (e.g. to 4 min or increased by 5 min respectively).

Poor hybridization/denaturation of probe/sample

Denaturation times and temperatures can be increased and hybridization times increased to increase signal intensity. Hybridizing samples for more than 16 hr can increase signal intensity. The authors regularly hybridize samples for 48-72 hr.

Sub-optimal filters

The filters used for viewing FISH signals can have a significant effect on signal intensity and one should avoid filters that are not specifically recommended by the probe manufacturer. The light transmission properties of filters also degrade over time so that they will eventually need to be replaced. Also ensure that the microscope objectives are suitable for viewing fluorescence.

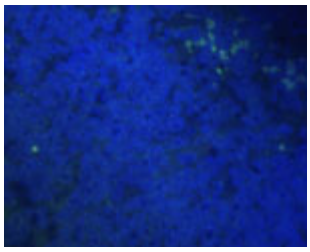
Sub-optimal microscope bulb
Mercury vapor bulbs degrade over time and should be replaced every 200-300 hr of usage. We recommend a HBO 100 mercury bulb in preference to a HBO 50 bulb, since this will significantly increase the signal intensity.

Problems with the probe

Ensure that probe components are brought to room temperature and thoroughly mixed prior to preparation and usage. Abbott probes may be prepared according to the manufacturer's instructions and stored at -20°C until required.

Air bubbles

It is possible to inadvertently trap air bubbles under the coverslip after applying the probe and this will result in weak or no FISH signals. These can be avoided by tapping the coverslip gently after it has been lowered onto the probe solution. If air bubbles do form, they can be removed by applying pressure gently to the surface of the coverslip, thereby pushing them to the edge. Air bubbles may also form if the surface of the section is not perfectly flat. In this case the coverslip can be pushed off the ridge of tissue onto a flatter area. If this is not possible the coverslip can be removed and the probe replaced by a greater volume (e.g. 2 µl instead of 1.5 µl). Alternatively small ridges of tissue can be removed by gently scraping with jewellers' forceps.



Weak FISH signals
Occasionally FISH signals will be too weak to interpret, possibly due to poor tissue fixation. It is worth repeating the analysis with increased demasking and pepsin incubation times to improve signal intensities. Longer hybridization times may also help.

A larger version of this image can be viewed by clicking on the image available at
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Reference

Srinivasan M, Sedmak D, Jewell S: Effect of fixatives and tissue processing on the content and integrity of nucleic acids. Am J Pathol, 2002, 161:1961-1971.

Acknowledgments

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