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Multi-colour FISH analysis of gene expression in formalin-fixed paraffin wax-embedded tissue

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There has been considerable interest in the *in situ* detection of mRNA, since this enables expression of specific genes to be placed within the context of an individual cell. The use of fluorescence-labelled probes (Ref. 1) has allowed more than one mRNA species to be targeted in cryostat sections (Ref. 2) and in cultured cells (Ref. 3). Paraffin wax-embedded material has, however, been more difficult to work with owing to problems with autofluorescence, but one report has indicated that it is possible to detect a single mRNA species in this material (Ref. 4). The ability to analyse gene expression in formalin-fixed paraffin wax-embedded material is important because it would make available an enormous resource of archival material. This is particularly relevant for studies designed to evaluate gene expression as a prognostic marker in tumours, since the expression data obtained from material collected several years previously could be correlated with the subsequent medical histories of the patients.

We have now extended previous work (Ref. 4) and describe here a protocol for the simultaneous detection of multiple mRNA species in formalin-fixed paraffin wax-embedded sections using small oligonucleotides. So far we have successfully used this approach to analyse gene expression in samples up to 7 years old and are confident that much older material could also be studied in this way. The use of a cooled CCD camera and associated software for the detection of signal provides a high degree of sensitivity. This method also allows for Haematoxylin & Eosin (H & E) staining to be carried out on the same slide, after the detection and capture of the fluorescence images.

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Table 1. Nucleotide sequences of oligonucleotides used as *BCL-2* probes

Oligo code	Sequence (5′–3′)	T_m (°C)
BCL2A	CCACTCGTAGCCCCTCTGCGACAGCTTAT	74.4
BCL2B	GTTGACGCTCTCCACACACATGACCCCA	76.1
BCL2C	CAGTCATCCACAGGGCGATGTTGTCCAC	75.5
BCL2D	GGTTCAGGTACTCAGTCATCCACAGGGC	71.4

Biopsy material was routinely fixed in 10% buffered formalin (pH 7.0) for 24 h. Sections of paraffin wax-embedded tissue were mounted by standard methods on microscope slides treated with 3-aminopropyltriethoxy-silane (APES). Oligonucleotide probes, 28–30 nucleotides in length, were 5′ labelled with either fluorescein isothiocyanate (FITC), rhodamine or digoxigenin. These were used either as a probe cocktail or as a single probe. Although the probe cocktails produced the stronger signals, it was still possible to detect mRNA with a single oligonucleotide probe (Fig. 1a). The CCD camera software is able to present separate images for each of the probes, or a composite of all three (Fig. la). The immunoglobulin kappa light chain gene probe (R & D Systems) BPR197 was a cocktail of eight oligonucleotides that were supplied prelabelled and the *BCL-2* proto-oncogene probe was designed by us (Table 1, sequence BCL2A) and supplied prelabelled (Oswell DNA Service, Southampton, UK). The T_m for each of the immunoglobulin probes was given as 90–92°C for the kappa probe and 92–94°C for the lambda probe, however, using MacVector software we calculated the T_m for the

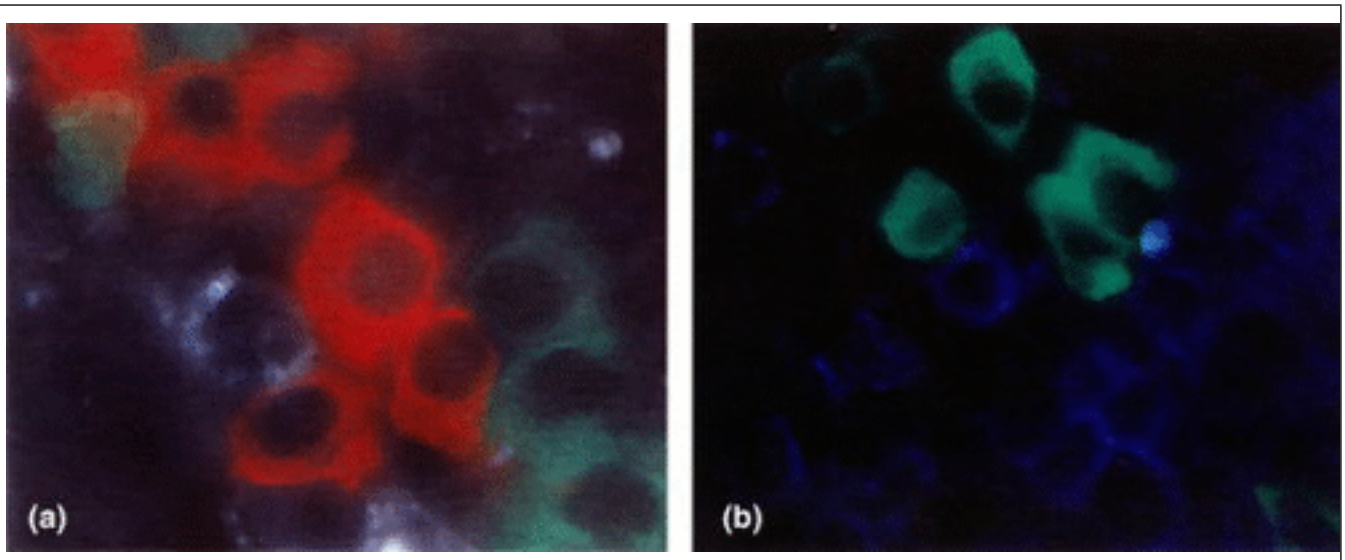

FIGURE 1. (a) 3 μm paraffin section of formalin-fixed human tonsil hybridized with oligonucleotide probes for immunoglobulin kappa light chain mRNA (FITC labelled, appearing green), immunoglobulin lambda light chain gene mRNA (Rhodamine labelled, appearing red) and *BCL-2* mRNA (Digoxigenin labelled, detected with anti-Dig AMCA, appearing blue). The kappa and lambda light chain immunoglobulin gene probes were both cocktails of oligonucleotides, but the *BCL-2* probe was a single oligonucleotide. The image was captured with a cooled CCD camera (Photometrics) attached to a Axioskop microscope (Zeiss) and processed with Smart Capture software (Digital Scientific) to produce the three-colour composite image. (b) Hybridization of an AMCA-labelled anti-T-cell-receptor antibody (blue) and a FITC-labelled probe for kappa light chain immunoglobulin MRNA (green) to a paraffin section of formalin-fixed human tonsil.

BCL-2 probe to be 74.4°C, which might indicate that this technique is fairly robust. Although the reduced signal for *BCL-2* (Fig. 1a) could be due to it being a low abundance mRNA species relative to those of the immunoglobulin genes, we have subsequently designed a cocktail of four *BCL-2* oligonucleotides (Table 1) and demonstrated a much stronger signal (data not shown). This indicates that relatively low abundance mRNA species should be detectable using this approach, a conclusion supported by our subsequent studies of expression of the *MYC* gene in high grade, non-Hodgkin's lymphoma. The use of oligonucleotides rather than antisense RNA probes has been suggested to reduce non-specific hybridization, thus leading to improved signal-to-background ratios (Ref. 5). The oligonucleotide probes are also much more stable. We normally store the labelled oligonucleotides, aliquoted in small volumes, in a light-proof container, at −20°C. As long as repeated freeze−thawing is avoided, we have been able to keep probes for over a year. So far, probe stability seems independent of the type of fluorophore used to label them.

One of the main problems with fluorescent *in situ* hybridization (FISH) analysis of paraffin sections is the high level of background signal. We have found, however, that signal resulting from the use of negative controls, such as sense oligonucleotides, RNAse pretreatment or probes for inappropriate genes (e.g. insulin which is not expressed in the tonsil) can allow an estimate of the background level to be made and thus enable the detection of genuine signal. We have also noted that the autofluorescence shown by paraffin sections diminishes significantly if the section is left at 4°C in the dark for a week after carrying out the hybridization experiments and prior to analysis. We have noted that because of the different protocols that are used in the fixation of biopsy tissue, proteinase K treatment is occasionally insufficient and a pepsin treatment has to be used instead. The latter approach is suitable for oligonucleotide hybridizations but appears to destroy protein epitopes and so antibody studies can only be carried out on serial sections.

The morphology of the cells could be demonstrated by H & E staining of the same slide after recording the FISH data. We have also found that staining sections with fluorescence-labelled antibodies can provide further useful protein marker information (on proteinase-K-treated sections) and can be used in conjunction with the oligoprobes (Fig. 1b). Together, these approaches provide a powerful analysis of archival material.

Protocol

Specimen preparation

1 Paraffin sections (3 μm) were dewaxed with xylene (2×5 min) and cleaned with 100% ethanol (2×5 min) followed by 95% ethanol (2×5 min). Slides

were washed sequentially in water (5 min), phosphate-buffered saline (PBS) (3 min), 0.3% Triton X-100 (15 min) and PBS (5 min).

2 Slides were incubated with 50 mM Tris, pH 7.6 (5 min), then 20 μg/ml proteinase K for 36 min at 37°C, followed by a 1-min incubation in 0.2% glycine and 2 × 5 min washes in PBS. [Alternatively, the slides were incubated with 0.01 M HCI at room temperature (5 min), then with 50 μg pepsin/ml 0.01 M HCI at 37°C (30 min), followed by 5 min incubation in 50 mM $MgCl_2$ (in PBS) and a 5 min wash in PBS].

3 Slides were post-fixed in 4% paraformaldehyde for 5 min and finally washed 2 × 5 min in PBS.

Hybridization and detection

1 Prepared sections were pre-hybridized with 300 μl hybridization solution (40% deionized formamide, 10% dextran sulphate, 0.5% SDS, 5 × SSC, Denhardt's solution, 1 mM EDTA, 0.3 ng ml yeast tRNA) in a humidified chamber for 1 h at 37°C.

2 After draining the slide, the oligonucleotide probes (each at a final concentration of 200 ng/ml) were added to the section in 55 μl hybridization solution and sealed under a 22 × 50 mm coverslip. Slides were heated for 10 min at 70°C on a heating block and then incubated overnight in a humidified chamber at 37°C. After careful removal of the coverslip, slides were washed in 4 × SSC (2 × 15 min), 2 × SSC (1 × 15 min) and deionized water.

3 Probes labelled with digoxigenin were detected immediately, before allowing the preparation to dry out. Preparations were blocked with SSCTM (4 × SSC, pH 7.0, 0.05% Tween 20, 5% dried milk powder) for 10 min at room temperature and then washed with SSCT (4 × SSC, pH 7.0, 0.05% Tween 20) for 3 min at room temperature. The slides were incubated with anti-digoxigenin-AMCA (Boehringer Mannheim), diluted 1:50 in SSCTM, at 37°C for 30 min in a humidified chamber, then washed in SSCT (3 × 3 min) and PBS (2 × 5 min) before being air dried and mounted in AF1 antifade (Citifluor Ltd).

4 After image capture, H & E staining could be applied by standard methods after an initial wash in 100% ethanol to remove the mountant.

5 When immunocytochemistry was also required, this was carried out immediately after the ethanol washes, but prior to probe hybridization.

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Products Used

immunoglobulin kappa light chain gene probe: immunoglobulin kappa light chain gene probe from R & D Systems
Anti-digoxigenin antibody: Anti-digoxigenin antibody from Boehringer Mannheim
antifade: antifade from Oncor Inc
CCD camera: CCD camera from Photometrics
CCD camera: CCD camera from Photometrics
Axioskop: Axioskop from Carl Zeiss
microscope (Stemi 2000): microscope (Stemi 2000) from Leica Microscopy & Scientific Instruments Group
Axioskop: Axioskop from Carl Zeiss
microscope: microscope from Carl Zeiss
Smart Capture software: Smart Capture software from Digital Scientific Ltd