Occasional articles

In situ hybridisation in perspective

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Introduction

As one of the nucleic acid assays, in situ hybridisation (ISH) permits the specific identification of genes and gene expression. It is, however, unique among these methods in providing this information without first removing the target nucleic acid sequence from its topographical surroundings (figure).

ISH was first described 21 years ago at the end of a decade in which most of the now commonly used hybridisation methods were also first reported.^{1,2} The technique's potential was quickly realised and within a few years it had been used for the identification of genomic,³ viral,⁴ and mRNA⁵ sequences in cryostat,⁴ paraffin wax,⁶ and electron microscopic preparations.⁷ Problems with probe preparation, however, hindered the general exploitation of ISH. Probes had to be prepared using crude isolates of nucleic acids, only radioactive labels could be used, and no routine method for the in vitro introduction of the label into the nucleic acid sequences was available. These problems were solved through the application of recombinant DNA technology,⁸ the synthesis of biotin UTP,⁹ and the description of the nick translation labelling method.¹⁰ Exploitation rapidly followed, and as with immunochemistry in the 1970s, ISH became a major development area in cellular pathology in the 1980s.

Now that ISH has "come of age" it is appropriate to enquire as to whether the technology is ready for general use and if it will follow in the footsteps of immunochemistry and become an important investigative tool. It is with these questions in mind that this paper has been written.

Technology

The principles and practice of ISH have been extensively reported¹¹⁻¹⁴ and should be referred to for a grounding in the technology. The scope of this discussion is to highlight aspects of ISH which determine the extent to which it can be regarded as a routine laboratory method.

Nucleic acid probes are to ISH what primary antibodies are to immunochemistry and may be simply defined as labelled nucleic acids. Double stranded (ds) and single stranded (ss) nucleic acids may be generated using recombinant DNA technology¹⁵ and ss oligonucleotides using synthesisers.¹⁶ The relative merits of these sequences are summarised in table 1. In-house production and quality assessment is a highly skilled undertaking. It

requires the availability of specialised equipment and is therefore likely to remain the domain of research institutions. An increasing range of unlabelled and labelled nucleic acids are now commercially available and the acquisition of suitable probes is therefore unlikely to present major difficulties.

For safety reasons only non-radioactive ISH is suitable for general laboratory use. Comparison of ³⁵S and ³H with biotin methods has shown that optimised non-radioactive methods can give results of equivalent¹⁷ or enhanced sensitivity.¹⁸⁻²⁰ For certain probe/target combinations, however, radioactive methods are preferable.^{20,21}

Principles of in situ hybridisation. A labelled nucleic acid sequence (1) hybridises through complementary base pairing to an immobilised target sequence and (2) the hybrid is detected by visualising the label (3).

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Table 1 Comparison of recombinant DNA and oligonucleotide nucleic acid probes

	Recombinant sequences		
	ds DNA	ss RNA	Oligonucleotides
Main advantage	Long sequences can be produced	High hybridisation efficiency	High hybridisation efficiency
Main disadvantage	Poor hybridisation efficiency	Difficult to produce	Insensitive unless used as "cocktails" of several labelled sequences

The availability of biotin UTP has largely been responsible for the rapid development of non-radioactive ISH. Biotin is present, however, as an endogenous component of several organs^{22,23} and is often present in active cells. Although methods for the reduction of interference due to endogenous biotin have been published,²³⁻²⁵ none seems entirely suitable. Several alternative labels have recently been described. Appropriately used they are of equivalent sensitivity to biotin but have no endogenous tissue distribution. Of these, digoxigenin UTP^{26,27} can be substituted directly for biotin UTP in labelling reactions. The other labels²⁸⁻³¹ are haptens which can be included into nucleic acids using chemical incorporation methods. These systems will probably replace biotin within this decade.

ISH for the detection of DNA targets may be applied to most standard histological³²⁻³⁴ and cytological³⁵⁻³⁷ preparations. Adequate fixation is essential but extensive cross linking, such as that induced by glutaraldehyde, should be avoided. Routine histological preparations³⁸ are often also suitable for mRNA detection. Many mRNA species, however, are quickly degraded and therefore prompt preservation either by freezing or fixation is required and, apart from the brain,³⁸ post mortem samples are seldom suitable.

The technique of ISH is analogous to immunochemistry but requires greater care in the preparation of solutions, most of which are required to be nuclease free, and is a complex operation. Similarities between the methods include pretreatments^{39,40} to expose target sequences and the use of detection systems⁴⁰⁻⁴³ to identify "probe" interactions. In contrast to the primary antibody step of immunochemistry, however, a necessarily complex hybridisation solution is required to ensure the specific interaction of the probe with the target nucleic acid. Some probes are

Table 2 Controls

Criteria	Control
Probe specificity	*Hybridisation with extracted target Nuclease digestion of target *Competitive absorption with unlabelled nucleic acid *Vector only hybridisation
Background	†Omit probe
Reproducibility	†Positive preparation
Sensitivity of hybridisation	Hybridise to filter bound serial dilutions of extracted target
Sensitivity of detection	Detect filter bound serial dilutions of probe

*These controls may not be required if probes are obtained commercially.

†These controls are required for each ISH run.

Table 3 Equipment requirements

Equipment	Use
Incubator, range 37-100°C	Proteolytic digestion, denaturation of DNA and hybridisation
Heating block	Denaturation of DNA probes
Fume hood	Handling of toxic solutions
Autoclave	Preparation of nuclease free solutions

commercially available in hybridisation solutions supplied alone or as part of ISH kits that render the method easier to undertake. It is essential, however, that the solution is tailored for each probe or target, that appropriate post-hybridisation washes are applied, and that controls are used to monitor specificity of hybridisation (table 2).

In contrast to the preparation of probes, the costs of performing ISH are relatively modest. Basic equipment requirements are listed in table 3. The most expensive reagents—proteolytic enzymes, hybridisation and detection solutions—are consumed in small volumes, typically at or below 50 µl per sample and are thus of minimal cost on a case by case basis. The technique is, however, labour intensive and therefore batch analysis should be undertaken to minimise expenditure. It is highly probable that automated systems⁴⁴ will be marketed, within the decade, allowing for further overall cost reductions.

From the foregoing account it is clear that the technology associated with ISH is ready for general use, but the extent to which it will eventually be adopted will depend on its usefulness as an investigative tool.

Applications

VIRAL

Viruses have traditionally been identified using culture methods. The technique is sensitive but slow and not applicable to all virus types. Hybridisation and immunochemical methods offer more rapid and equally sensitive alternatives. The most sensitive of these is undoubtedly the polymerase chain reaction⁴⁵ which provides an accurate assessment of prevalence of infection. Due to their "lower" sensitivities, however, dot⁴⁶ and Southern blotting methods may be more suited to differentiating latent from active infection. Southern blotting is also particularly useful for the study of integration of viral genomes into tumour cells.⁴⁷ None of these methods, however, provides simultaneous morphological information and for this either immunochemistry⁴⁸ or ISH must be used. In general these methods are of comparable sensitivity,⁴⁵ however, in certain instances—for example, where antigen is absent, or where strain specific antisera are not available—ISH is the preferred method.

Apart from the identification of cytomegalovirus (CMV) infection⁴⁹ and definition of human papilloma virus (HPV) in cervical lesions,⁵⁰ ISH is presently little used for diagnostic purposes. In contrast, the technique is

extensively used in research into the pathogenesis of viral infection and its association with cancer.

ISH has provided a valuable insight into the cell types associated with latent and productive viral infection. Examples include hepatitis B virus in liver cells,⁵¹ CMV in lung⁵² and in brain, together with human immunodeficiency virus (HIV).⁵³ JC virus in the central nervous system⁵⁴ and B19 parvovirus in a variety of fetal tissues.⁵⁵ ISH has also provided evidence of viral involvement in other non-malignant processes. Examples include the demonstration of CMV in retinopathies of patients with AIDS⁵⁶ and HSV-1 infection in psychiatric illness.⁵⁷

Specific subtypes of HPV are associated with a wide variety of benign and malignant epithelial lesions.⁵⁸⁻⁶³ In contrast to immunochemical analysis, ISH permits the identification of HPV subtypes and has been successfully used for retrospective analysis⁶⁴ for which other hybridisation assays are often unsuitable. The technique also provides the most convenient method for the clinical assessment of HPV state in cervical punch biopsy specimens.

The association of Epstein-Barr virus (EBV) with infectious mononucleosis, Burkitt's lymphoma, and nasopharyngeal carcinoma is well established. Recent hybridisation studies have shown a more general association of this virus with lymphomas, including Hodgkin's disease,⁶⁵ B cell lymphoma,²¹ nasal T cell lymphoma,⁶⁶ primary central nervous system lymphoma,⁶⁷ and lymphoepithelioma-like carcinoma of the lung.⁶⁸

Interestingly, immunosuppression and AIDS have been features in some of the lymphoma reports,^{21 67} and the association of EBV with oral hairy leucoplacia in patients with AIDS has been confirmed by ISH.⁶⁹ Research interest in EBV and haemopoietic disorders will probably continue, but due to the low copy number of genomic sequences radioactive ISH²¹ methods are sometimes required to ensure detection of the virus.

GENOMIC

Radioactive ISH techniques applied to karyotype preparations have provided valuable information on chromosomal changes in tumours,^{70 71} viral infection,⁷² and hereditary diseases.⁷³ The development of fluorescent ISH methods⁷⁴ of equivalent sensitivity may, however, open this area for more general study. Recent reports also indicate that, by the use of multiple hapten probes, chromosomal analysis of interphase nuclei can be readily achieved in dispersed or intact tumour populations.^{37 75} Using these methods, problems due to changes in cell populations during karyotype preparation are avoided⁷⁵ and greater information is provided on the heterogeneity of chromosomes in individual cells compared with that obtained by FACS analysis.⁷⁶

Whole genomic probes and probes for repetitive sequences on the Y chromosome locate large targets and are therefore suitable for use in the development of non-radioactive ISH protocols.^{14 40} They also have important

pathological applications. Genomic probes have been used to elucidate the species of cells in xenografts,⁷⁷ while demonstration of the Y chromosome provides unequivocal pre⁷⁸ and postnatal sex determination and a means of monitoring after bone marrow transplantation when donor and recipient are of the opposite sex.⁷⁹

MESSENGER RNA

As the protein products of mRNA can often be shown immunochemically the question must be asked as to what advantages there are in using ISH for the demonstration of phenotypic targets? To an extent the answers depend on whether the investigation is for research or diagnostic purposes.

For research applications ISH provides unequivocal information on the cellular site of synthesis. It has been used to investigate whether Reed-Sternberg cells synthesise immunoglobulin⁸⁰ and to elucidate which cells produce albumin and collagen⁸¹ in the face of equivocal immunochemistry. ISH can also provide precise information on changes in mRNA synthesis in response to manipulation of the cellular environment. Examples are provided in the investigation of mRNA hormone synthesis alone,^{82 83} or in combination with immunochemical assessment of storage of products.⁸⁴ The combination of ISH and immunochemistry has also been used for developmental biology investigations,⁸⁵⁻⁸⁸ but for this the low copy number of mRNA sequences often requires the use of radioactive probes.

ISH for mRNA has yet to make an impact on diagnostic pathology. The intense reaction obtained when probes for poly A tailed mRNA³⁸ are used on paraffin wax sections suggests that many sequences are available for demonstration. Only a few potential probes, however, have been described.^{31 89}

ISH for mRNA will probably find a niche in diagnostic pathology, initially in areas where problems are experienced with immunochemistry. In situations where protein is rapidly exported from a cell—for example, in APUDomas or where cells and tissues are bathed in the same protein such as myelomas—⁹⁰ ISH will be of potential use. Furthermore, it is conceivable that when epitopes are pathologically changed that unaffected portions of the mRNA will be available for demonstration. Further studies are clearly required and, in particular, comparisons of ISH and immunochemistry⁹¹ need to be undertaken. Based on these results situations in which ISH is advantageous or complementary to information provided by immunochemistry should be identified.

Summary

In the introduction to this review two questions were posed: is the technology associated with ISH ready for general use, and will the method become an important investigative tool? With the exception of the demonstration of some single and low copy sequences, non-radioactive

ISH is now sufficiently developed and simplified to make it a routine technique. It is also clear that ISH will continue to have an important research role. In diagnostic pathology the technique is already providing valuable information and the present decade should see the development of many more diagnostic applications.

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