Demystified . . .

This journal is continuously expanding and evolving. This article is the first of a new series of reviews intended to "demystify" techniques and concepts in molecular biology and pathology. The aims of this series are to take a particular topic and to clarify what is often a complex field. Conventional reviews often assume a certain level of knowledge, not least a familiarity with the jargon associated with the subject.

We have been approached by readers who expressed a wish for review articles that go back to basics. Even those who are experienced in their own field may find themselves baffled by specialised articles. We can all benefit from being guided through fundamental principles and it is hoped that this new series will fulfil this role.

We welcome suggestions by readers for subjects to be covered in this series, which we hope will increase the value of this journal.

JOHN CROCKER
DAVID BURNETT
Editors, Molecular Pathology

In situ hybridisation

C S Herrington

In situ hybridisation is a useful technique for the demonstration of nucleic acids in conjunction with morphological detail. This review describes the basic components and principles of the technique and gives a brief overview of its applications.

Basic principles

In situ hybridisation can be defined as the morphological localisation of genetic sequences. The objective therefore is to determine the presence or absence of specific DNA or RNA species and to localise those species to particular cellular or chromosomal sites. The identity of sequences within cells is established by exploiting a fundamental property of nucleic acids; their ability to anneal to one another in a sequence specific manner to form hybrids. This is true not only of the two strands of DNA but also of RNA–DNA and RNA–RNA combinations. By labelling one of these two strands, the hybrids formed by such annealing can be detected by a variety of means. The basic requirements of the technique are therefore: a probe that is specific for the sequence of interest and is labelled to allow appropriate detection; and preservation of sufficient morphological detail to allow identification of the location of the labelled probe.

Technical elements

In situ hybridisation techniques have four components (fig 1):

- sample preparation
- probe preparation and labelling
- denaturation and hybridisation
- post-hybridisation washing and detection.

This account will not include technical details, which can be found elsewhere.[1–3]

SAMPLE PREPARATION

A wide variety of cellular material can be analysed by in situ hybridisation and each sample type requires specific pretreatment measures. Thus, individual cells obtained from cell cultures, from exfoliated or aspirated clinical samples, or by disaggregation of fresh tissue require fixation to preserve morphological detail. Precipitating fixatives such as 70% ethanol[4] and the conventional fixative used in cytogenetics (methanol:acetic acid; 3:1) are very useful as they allow cell suspensions to be stored indefinitely at −20°C. Moreover, in situ hybridisation can often be performed directly on such fixed cells or with relatively mild proteolytic digestion. Fixation of isolated cells in cross linking fixatives such as neutral buffered formalin has been suggested to improve morphology further but this is at the expense of the requirement for more rigorous proteolysis. Fixation under controlled circumstances has the added advantage that variability is reduced to a minimum. This is particularly important when proteolytic digestion conditions are being optimised.

Archival paraffin wax embedded tissues are an almost limitless source of material for study using a variety of molecular techniques. This type of material is particularly useful for in situ hybridisation as the morphology of the tissues is optimally preserved. Most routine laboratories use either neutralised or neutral buffered formalin for tissue fixation. These fixatives preserve nucleic acids relatively well. Other fixatives, however, such as Bouin's (used for fixation of testicular biopsies), preserve nucleic acids poorly. For most studies, the fixation and embedding processes simply have to be accepted and the subsequent elements of the procedure optimised for the particular specimens being used.

Pretreatment of cells and tissues

Once the cells or tissues have been fixed, the nucleic acids contained within them have to be made available to the probe, a process known as unmasking. The mainstay of this process is proteolytic digestion, most frequently using proteinase K or pepsin HCl, although many other enzymes have been used. Proteolysis removes components of the cell nucleus and cytoplasm to allow probe access. Thus, underdigestion leads to suboptimal probe penetration. Conversely, overdigestion leads to destruction of cell and tissue architecture. This results in loss of both nucleic acids and morphological detail. In both these situations, there is relative failure of the hybridisation reaction as probe and target nucleic acid are not brought together under optimal conditions. Sometimes, acceptable results cannot be obtained despite optimisation of proteolytic digestion. In this situation, coupling of digestion to other unmasking techniques such as treatment with sodium bisulphite, sodium thiocyanate or hydrochloric acid, or microwave pretreatment can be used to improve probe access while retaining morphological detail.[5] The mechanisms by which these steps improve nucleic acid unmasking are not clearly understood, although extraction of histone proteins from nuclei is thought to be one important

Department of Pathology, University of Liverpool, Duncan Building, Daulby Street, Liverpool L69 3GA, UK
C S Herrington

Correspondence to:
Dr Herrington.
email: c.s.herrington@liv.ac.uk

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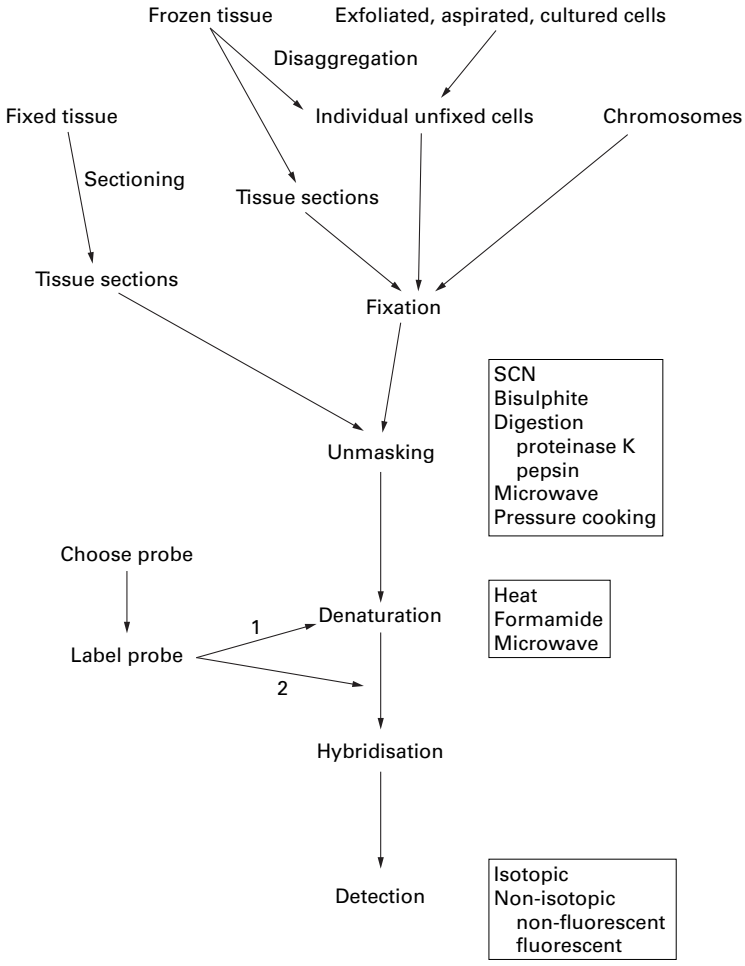

Figure 1 Overview of in situ hybridisation. 1, co-denaturation; 2, separate denaturation.

factor. Following unmasking, postfixation, particularly in aldehyde based fixatives such as paraformaldehyde, may be useful for the prevention of loss of material from the slide and for further preservation of morphology. Following proteolysis, many workers dehydrate their preparations using an ethanol series. This leads to some improvement in morphology particularly in cellular preparations although, for paraffin sections, dehydration by air drying seems just as effective.

PROBES

A wide variety of probes can be used for in situ hybridisation and the appropriate type is determined to a large extent by the application.[5] For DNA detection (for example, DNA viruses, chromosome specific probes or locus specific probes[6]), double stranded DNA probes are the most commonly encountered. Many of these are now commercially available either as individual probes or in kit form. For RNA detection, RNA probes (riboprobes) are often used because: RNA–RNA hybrids are more stable than DNA–RNA hybrids; riboprobe vectors can be used to generate both sense and antisense probes to allow control of hybridisation; and unbound probe can be digested using RNAse, which does not digest double stranded RNA. Alternatively, oligonucleotides, either singly or in a cocktail, can be used, particularly for the detection of high abundance RNA. These have the advantage that they can be custom synthesised and have high specificity. They can also be labelled efficiently by 3' tailing by which multiple labelled nucleotides can be added enzymatically to each oligonucleotide molecule.

The choice of label is governed to some extent by personal preference and, in some situations, commercial availability. Isotopic (radioactive) labelling is now largely confined to RNA detection, particularly where non-isotopic alternatives are unsuccessful. ^{35}S is the most popular label as it combines reasonable specific activity with relatively high morphological resolution. Many laboratories now use non-isotopic labels exclusively. The most commonly used of these are biotin and digoxigenin but many others—for example, fluorescein, are available.[7] One advantage of using a fluorochrome as hapten is that both direct fluorescent detection and indirect immunoenzymatic detection can be used for the same probe. The flexibility of these non-isotopic labels, coupled with their high morphological resolution, has led to a dramatic increase in their use for in situ hybridisation.

Labelling of probes is most commonly performed using enzymatic methods. DNA probes are conveniently labelled by either nick translation or randomly primed extension, both of which introduce labelled nucleotides such as digoxigenin-11-dUTP into the probe molecules. RNA probes are generally labelled by in vitro transcription whereby the probe sequence is cloned into a vector containing RNA polymerase promoter sites, and probe molecules generated using 'phage RNA polymerase mediated incorporation of labelled nucleotides such as digoxigenin-11-UTP. A variety of polymerases can be used, including T3, T7, and SP6. Oligonucleotides can be labelled during synthesis, when 5' labelled nucleotides can be incorporated directly into the molecule. Alternatively, enzymatic procedures can be used for either 5' or 3' labelling. One advantage of 3' end labelling is that multiple probe labels can be introduced to form a "tail", thus increasing the sensitivity of detection. Finally, probe labelling can be carried out using polymerase chain reaction (PCR) based methods, with either direct incorporation of labelled nucleotides or by using labelled primers. Details of the practical details of probe labelling can be found elsewhere.[7]

DENATURATION AND HYBRIDISATION

Once the appropriate probe molecule has been isolated and labelled, and the target nucleic acid exposed within the cell or tissue of interest, the probe and target must be brought together in such a way that specific hybridisation—joining together—can occur. For DNA probes and targets, this requires initial separation of the double stranded nucleic acid molecules into their single strands—denaturation. This can be achieved either by chemical means or by heating the double stranded DNA to above its melting temperature, either in an oven or using a microwave. In

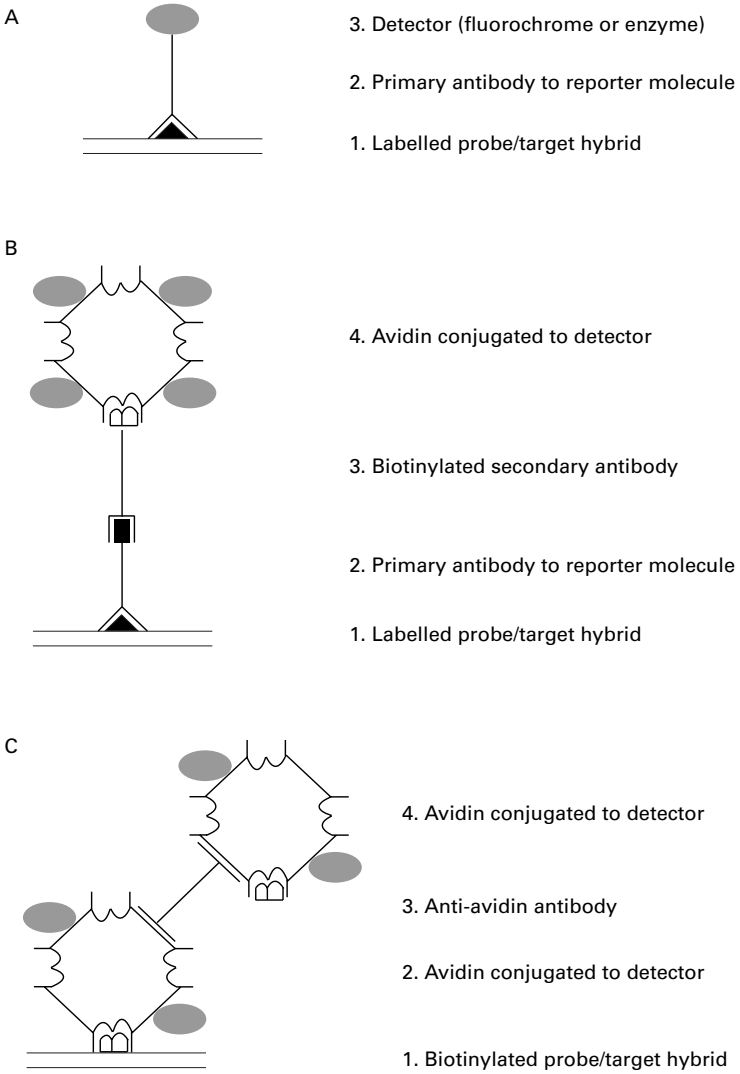

Figure 2 Examples of commonly used detection systems. (A) Direct detection of probe label using a primary antibody conjugated to a detector, such as a fluorochrome (for example, FITC) or an enzyme (for example, horseradish peroxidase). (B) Indirect detection of probe label using a biotinylated secondary antibody and an avidin conjugate. This conjugate can be labelled with either a fluorochrome or an enzyme to allow fluorescent or non-fluorescent detection. (C) An avidin based sandwich technique allowing high sensitivity detection of biotinylated probes. Two layers of an avidin conjugate are linked using an anti-avidin antibody.

reality, a combination of these two approaches is used. If double stranded DNA is heated to a temperature above its melting temperature (which is determined by both the length and sequence of the DNA), the two strands separate. The temperature at which this occurs can be altered by the inclusion of organic solvents in the denaturation–hybridisation solution. The most commonly used of these is formamide, which destabilises the double stranded structure of DNA at a given temperature, thus reducing the effective melting temperature of the hybrids. This reduces the need for high temperature incubation and consequently leads to better preservation of morphology.

For DNA detection using DNA probes, both probe and target molecules must be denatured. This can be achieved in two ways—that is, separately or by co-denaturation. The choice between these two methods is largely one of personal preference, although separate denaturation is most commonly used in molecular cytogenetics for fluorescent in situ hybridisation (FISH) and co-denaturation is most commonly used in the analysis of tissue sections. The main advantage of co-denaturation is that the number of practical steps is reduced. However, some argue that morphological preservation is less optimal than with separate probe and target denaturation. Riboprobes and oligonucleotide probes are single stranded, as is cellular RNA. Therefore, denaturation is not essential for RNA detection. Despite this, some workers have reported that denaturation improves the sensitivity of riboprobe detection of RNA, possibly by removing the secondary structure of RNA probe and target, but this is not a universal requirement.

Once the probe and target molecules have been rendered single stranded, all that is required for annealing to take place is for either the probe and target molecules to be brought together (for RNA detection and for separate denaturation of DNA), or for the incubation temperature to be reduced to below the melting temperature of the required hybrids (for DNA detection by co-denaturation). At this point, the specificity (or stringency) of the hybridisation reaction is determined. Thus, if hybridisation is carried out at too high a temperature, no probe annealing occurs as the probe and target remain single stranded. If hybridisation is carried out at too low a temperature, probe and target molecules that are not perfectly matched will be allowed to anneal, thus reducing the specificity of the reaction. The appropriate hybridisation temperature is determined by experiment.[8] Alternatively, the same effect can be achieved by altering the chemical constitution of the hybridisation solution while keeping the temperature constant. Thus, increasing the formamide concentration (which destabilises mismatched hybrids) has the same effect as hybridisation at a higher temperature. Other parameters that affect the specificity of the reaction are: the concentration of monovalent cation (usually Na^+); the length of the probe molecules; and the probe concentration. Thus, a reduction in salt concentration increases the specificity of the reaction, as does lengthening the probe. Increasing the probe concentration drives the reaction in favour of the formation of probe–target hybrids thus speeding the reaction up but may also lead to non-specific background staining. Generally, a probe concentration of 1–2 ng/μl is optimum; again, this is best determined by experiment. Hybridisation time is also generally determined by experiment. However, as a rule, the time required is dependent on how repetitive the target sequence is: thus, highly repetitive targets, or targets present in high copy number, generally require short hybridisation times (two hours), whereas low copy number targets require overnight hybridisation.[5 8 9]

DETECTION OF HYBRIDS

Following hybridisation, the first step is to remove unbound hybridisation reagents. This

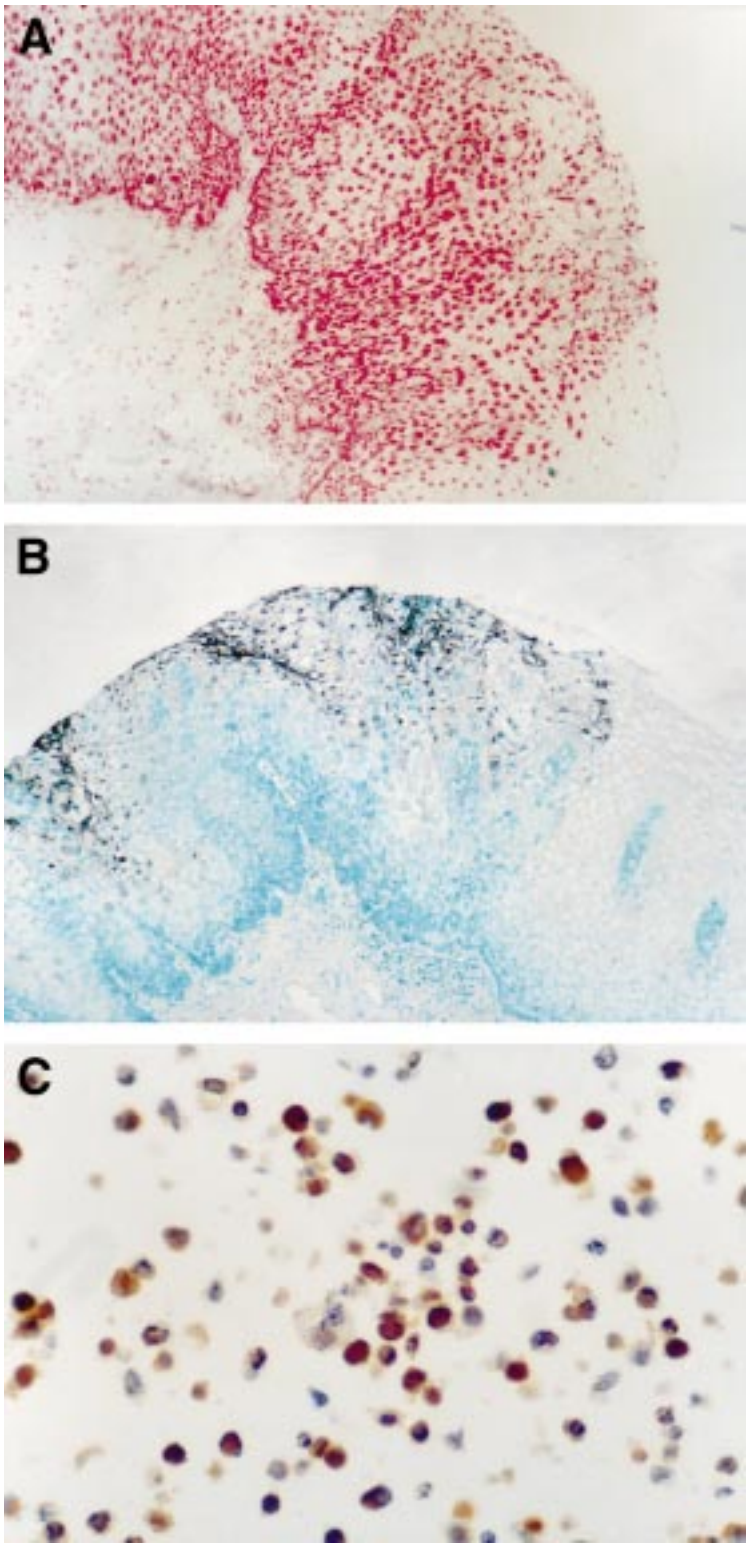

Figure 3 Representative examples of DNA detection by non-fluorescent in situ hybridisation. (A) A probe for total human DNA demonstrates all of the nuclei (red) in a strip of cervical squamous epithelium and underlying stroma. The probe label (biotin) was detected using a two stage alkaline phosphatase based system (streptavidin followed by biotinylated alkaline phosphatase) with naphthol AS-MX phosphate/Fast Red as chromogenic substrate. (B) Human papillomavirus DNA is identified as a black deposit within squamous epithelial cell nuclei in a parallel section of the biopsy shown in (A) using an immunogold silver solution method. (C) Herpes simplex virus is localised within infected cultured cells (brown) using an avidin biotinylated peroxidase complex (ABC) method with diaminobenzidine (DAB) as chromogenic substrate.

is generally carried out in a saline solution in which probe–target hybrids are stable (standard saline citrate; SSC). At this point, the specificity of the reaction can again be manipulated, although only an increase in specificity is possible at this stage. Thus, washing in solutions containing lower salt concentrations, or higher formamide concentrations than the hybridisation solution, at a higher temperature than that at which hybridisation was carried out, increases specificity by dissociating imperfectly matched hybrids. Examples of this are: hybridisation in 60% formamide, 2× SSC at 37°C followed by washing in 60% formamide, 2× SSC at 42°C; and hybridisation in 50% formamide, 2× SSC at 37°C followed by washing in 0.1× SSC at 37°C.

Once the appropriate level of specificity has been achieved, the presence of probe–target hybrids can be demonstrated by detection of the probe label molecules. For isotopic labels, this is achieved by using dip-slide emulsion techniques. For non-isotopic labels, there are two basic choices: fluorescent or non-fluorescent detection. Probes labelled directly with either fluorochromes or enzymes can be detected directly by immediate fluorescence microscopy and by incubation in substrate solution, respectively. More commonly, indirect detection methods are used, based on either affinity or immunodetection (fig 2). The avidin or antibody systems are then linked to either a fluorochrome or to an enzyme–substrate combination (figs 3 and 4). Fluorescence based approaches are particularly useful when high resolution is required or where more than one sequence is hybridised to the same cell or tissue preparation. The latter is particularly important in interphase cytogenetics where multicolour hybridisation techniques are used frequently. Non-fluorescent methods are useful where accurate morphological correlation is required, particularly in the assessment of paraffin sections. Alkaline phosphatase based systems are generally more sensitive than those using peroxidase (fig 4) but peroxidase substrates tend to give greater resolution. The recently described amplification system based on peroxidase catalysed deposition of biotinylated tyramine[10] allows enhancement of the sensitivity of peroxidase based systems and can also be applied to alkaline phosphatase based detection.

Applications

The applications of in situ hybridisation can be considered on the basis of: the nucleic acid species being detected; its cellular localisation; and whether it is exogenous or endogenous in origin (fig 5). Endogenous DNA is largely nuclear—for example, chromosomes and individual genes, and these are most frequently detected by FISH,[6] which is be the subject of a further article in this series.[11] Non-fluorescent methods can also be used for larger targets and give good morphological correlation. Endogenous cytoplasmic DNA—for example, mitochondrial DNA, can also be examined but this is an uncommon application. Exogenous DNA is largely viral in origin and is almost

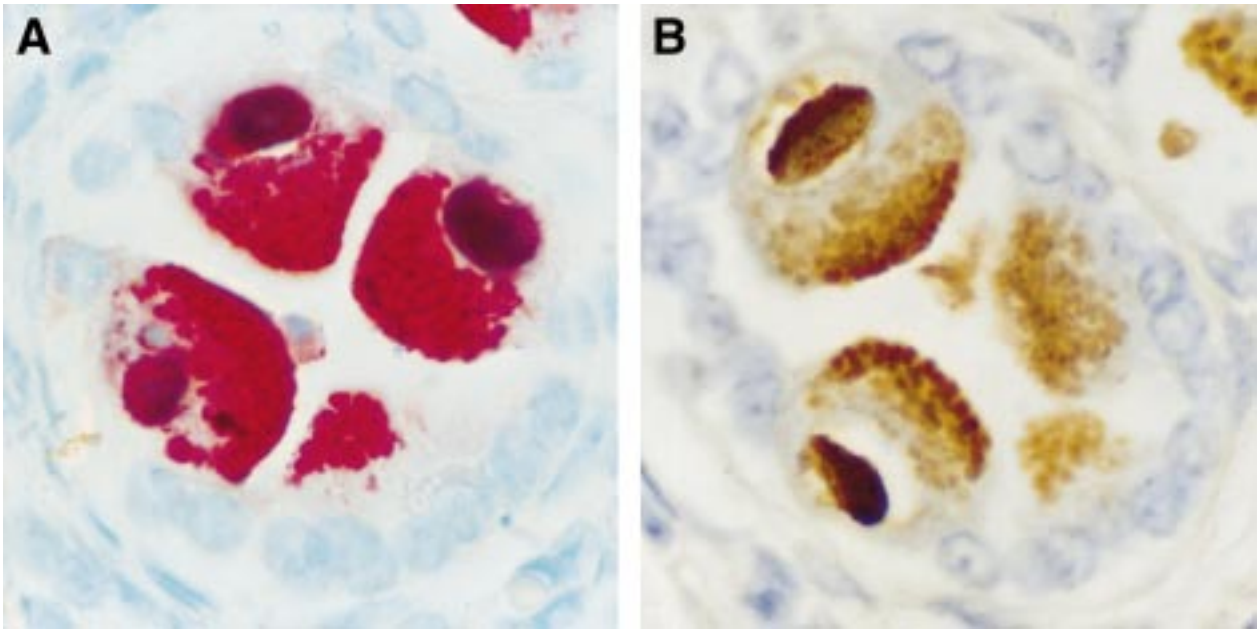

Figure 4 Comparison of peroxidase and alkaline phosphatase detection of cytomegalovirus in renal tubular epithelial cells. The signal is both more intense and more widespread when detected using alkaline phosphatase Fast Red (A) compared with a peroxidase ABC method (B). These detection systems are the same as those used in figs 3(A) and 3(C), respectively.

universally nuclear in distribution. The most extensively studied example is human papillomavirus DNA, which can be detected easily in routine biopsy material (fig 3) and in cervical smears using standard in situ hybridisation techniques.[8] Other DNA viruses that can be analysed by in situ hybridisation are: cytomegalovirus (fig 4),[12] herpes simplex virus (fig 3),[13] varicella zoster virus,[14] Epstein-Barr virus,[15] adenovirus,[16] and JC virus.[17] From a diagnostic point of view, many of these viruses are more appropriately detected using PCR based methodology but in situ hybridisation is of clear value when morphological localisation is required.

RNA sequences are generally located within the cell cytoplasm, the main exception being heterogeneous nuclear, or unspliced, RNA. The most common application of in situ hybridisation in this situation is for the detection of specific mRNA species.[18] This is of particular value for the demonstration of cytokine mRNA and mRNA from other secreted products as it demonstrates true cellular synthesis, excluding the possibility that the presence of protein simply represents absorption by the cell. Although RNA in situ hybridisation is clearly less sensitive than—for example, reverse transcriptase-PCR, morphological localisation of RNA sequences is often as important as the determination of their presence. For example, determination of the cells synthesising cytokine RNA is of fundamental importance in the study of cell–cell interactions. RNA viruses and the viral transcripts of DNA viruses can also be demonstrated by in situ hybridisation. Examples of RNA viruses are: parvovirus,[19] retroviruses,[20] measles,[21] and enteroviruses.[22] Perhaps the best example of the detection of transcripts from a DNA virus is the use of EBER RNA for the determination of Epstein-Barr virus infection.[23]

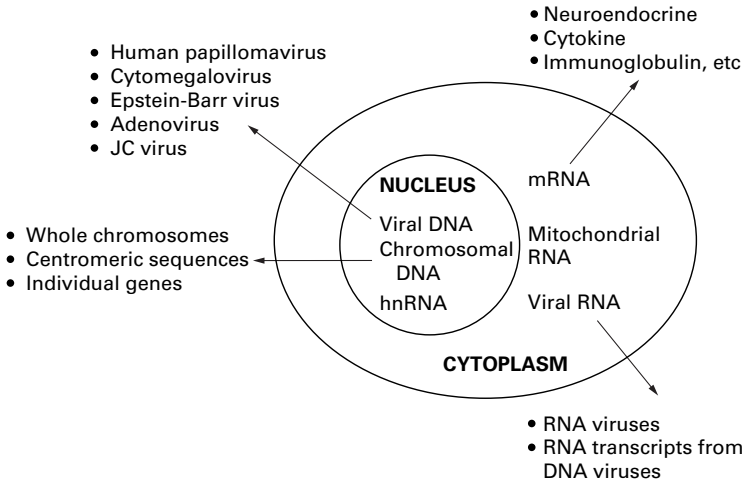

Figure 5 Applications of in situ hybridisation.

Conclusion

With modern technical refinements and the ready availability of many labelled probes from several commercial sources, in situ hybridisation is becoming a routinely applicable technique in both research and diagnostic laboratories. This is particularly true of FISH, which is extensively used in clinical cytogenetics. The strength of in situ hybridisation is its ability to localise nucleic acids to particular cells and to subcellular compartments, a property that is only rivalled by microdissection based techniques, which, with the exception perhaps of the recently described laser based microdissection approach,[24] are too crude for accurate analysis of populations of cells at the individual cell level. In situ hybridisation is of particular value where specific, morphologically orientated questions are being asked and, in this

situation, it provides a powerful adjunct to extraction based techniques.

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1 Herrington CS, McGee JO'D, eds. *Diagnostic molecular pathology: a practical approach*, Volumes 1 and 2. Oxford: Oxford University Press, 1992.
2 Levy ER, Herrington CS, eds. *Non-isotopic methods in molecular biology: a practical approach*. Oxford: Oxford University Press, 1995.
3 Wilkinson DG. *In situ hybridization: a practical approach*. Oxford: Oxford University Press, 1992.
4 Hopman AHN, Poddighe P, Moesker O, *et al*. Interphase cytogenetics: an approach to the detection of genetic aberrations in tumours. In: Herrington CS, McGee JO'D, eds. *Diagnostic molecular pathology: a practical approach*, Volume 1. Oxford: Oxford University Press, 1992:141–67.
5 Southern SA, Herrington CS. In situ hybridization. In: O'Leary JJ, Herrington CS, eds. *PCR in situ hybridization: a practical approach*. Oxford: Oxford University Press 1998: 27–51.
6 Klinger KW. Cytogenetic analysis. In: Levy ER, Herrington CS, eds. *Nonisotopic methods in molecular biology: a practical approach*, Oxford: Oxford University Press, 1995:25–50.
7 Hopman AHN, Speel EJM, Voorter CEM, *et al*. Probe labelling methods. In: Levy ER, Herrington CS, eds. *Nonisotopic methods in molecular biology: a practical approach*. Oxford: Oxford University Press, 1995:1–24.
8 Herrington CS, McGee JO'D. Principles and basic methodology of in situ hybridization. In: Herrington CS, McGee JO'D, eds. *Diagnostic molecular pathology: a practical approach*, Volume 1. Oxford: Oxford University Press, 1992:69–102.
9 Herrington CS, McGee JO'D. Discrimination of closely homologous genomic and viral sequences in cells and tissues: further characterisation of Tmt. *Histochem J* 1994; **26**:545–52.
10 King G, Payne S, Walker F, *et al*. A highly sensitive detection method for immunohistochemistry using biotinylated tyramine. *J Pathol* 1997;**183**:237–41.
11 Waters JJ, Barlow AL, Gould CP. Demystified . . . FISH. *J Clin Pathol: Mol Pathol*. [In press.]
12 Gerna G, Baldanti F, Zella D, *et al*. Detection of human cytomegalovirus DNA: how, when and where? *Scand J Infect Dis Suppl* 1995;**99**:11–15.
13 Obara Y, Furuta Y, Takasu T, *et al*. Distribution of herpes simplex virus types 1 and 2 genomes in human spinal ganglia studied by PCR and in situ hybridization. *J Med Virol* 1997;**52**:136–42.
14 Annunziato P, Lungu O, Gershon A, *et al*. In situ hybridization detection of varicella zoster virus in paraffin-embedded skin biopsy samples. *Clin Diagn Virol* 1996;**7**: 69–76.
15 Coates PJ, Mak WP, Slavin G, *et al*. Detection of single copies of Epstein-Barr virus in paraffin wax sections by non-radioactive in situ hybridisation. *J Clin Pathol* 1991;**44**: 487–91.
16 Ohori NP, Michaels MG, Jaffe R, *et al*. Adenovirus pneumonia in lung transplant recipients. *Hum Pathol* 1995; **26**:1073–9.
17 Boerman RH, Arnoldus EPJ, Raap AK, *et al*. Diagnosis of progressive multifocal leucoencephalopathy by hybridisation techniques. *J Clin Pathol* 1989;**42**:153–61.
18 Pringle H. Non-isotopic detection of RNA in situ. In: Levy ER, Herrington CS, eds. *Nonisotopic methods in molecular biology: a practical approach* Oxford: Oxford University Press, 1995:85–110.
19 Liu W, Ittmann M, Liu J, *et al*. Human parvovirus B19 in bone marrows from adults with acquired immunodeficiency syndrome: a comparative study using in situ hybridization and immunohistochemistry. *Hum Pathol* 1997;**28**:760–6.
20 Richman DD, McCutchan JA, Spector SA. Detecting human immunodeficiency virus RNA in peripheral blood mononuclear cells by nucleic acid hybridization. *J Infect Dis* 1987;**156**:823–7.
21 McMahon J, McQuaid S. The use of microwave irradiation as a pretreatment to in situ hybridization for the detection of measles virus and chicken anaemia virus in formalin-fixed paraffin-embedded tissue. *Histochem J* 1996;**28**:157–64.
22 Muir P, Nicholson F, Illavia SJ, *et al*. Serological and molecular evidence of enterovirus infection in patients with end-stage dilated cardiomyopathy. *Heart* 1996;**76**:243–9.
23 Lones MA, Shintaku IP, Weiss LM, *et al*. Posttransplant lymphoproliferative disorder in liver allograft biopsies: a comparison of three methods for the demonstration of Epstein-Barr virus. *Hum Pathol* 1997;**28**:533–9.
24 Becker I, Becker K-F, Röhrl MH, *et al*. Single-cell mutation analysis of tumours from stained histologic slides. *Lab Invest* 1996;**75**:801–7.