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Non-radioisotopic labels for *in situ* hybridisation histochemistry: a histochemist's view

Introduction

The early development of *in situ* hybridisation relied heavily upon the use of radioisotopes and autoradiography for the visualisation of specifically hybridised gene probes (Buongiorno-Nardelli & Amaldi, 1969; Gall & Pardue, 1969, 1971). Radioisotopic labelling retains an important rôle, especially where maximum sensitivity is required, but in recent years a group of techniques employing non-radioisotopic labelling technology have been developed. They have been designed to avoid the perceived drawbacks of radiolabels in terms of safety and inconvenience.

In this review I hope to provide a flavour of the range of non-isotopic techniques available, to discuss their relative merits and to divine future developments. Some general methodologies are outlined but these must be considered as general guidelines from which optimal protocols must be individually determined (some detailed protocols are provided in later chapters).

Aim

In situ hybridisation identifies specific DNA or RNA sequences in tissue sections. What questions can *in situ* hybridisation answer that other methods cannot?

Standard molecular biological methods using gel electrophoretic separation of restriction fragments is ideal for characterising genomes, for viral detection or general changes in gene expression. However, in certain respects these methods have severe limitations. Extremely small quantities of nucleic acid can be detected by filter hybridisation but the identity of the cell containing the target sequence is unknown. Therefore electrophoretic separation methods provide no distributional information but are rather an accurate measure of the average nucleic acid content of a tissue homogenate. Only *in situ* hybridisation provides the capacity for the identification of specific target sequences within a mixed cell population. This depends upon the presence of localised areas of high target sequence concentration within a background of non-homologous sequences. Particular examples may be seen

in the localisation of tissue-specific mRNA transcripts (Dirks *et al.*, 1989), viral (Burns *et al.*, 1988) or bacterial infection (Näher, Petzoldt & Sethi, 1988), oncogenes in small cell lung carcinoma (Gu *et al.*, 1988), sex determination (Burns *et al.*, 1985) or chromosomal abnormalities (Hopman *et al.*, 1988) and physical gene mapping (Bhatt *et al.*, 1988).

How is it done?

Essentially all hybridisations depend on the fundamental characteristic of nucleic acids; that is single strands of DNA, made up of complementary base sequences, bind together more tightly than non-homologous sequences. All hybridisation methods depend upon a thorough understanding of the physico-chemical forces which control hybridisation.

Types of nucleic acid hybrid:

DNA–DNA	Increasing stability
DNA–RNA	at a particular
RNA–RNA	temperature

Double stranded (DS) DNA, single stranded (SS) DNA, DNA oligonucleotides or RNA may be used as probes for hybridisation.

Factors affecting *in situ* hybridisation sensitivity

The factors affecting hybrid stability and hybridisation kinetics, and thereby sensitivity, have been well established for duplexes in solution, but there are important differences between hybrids in solution and as immobilised duplexes, particularly so in tissue sections. Final signal strength is determined by a number of interdependent factors:

1. Probe type: DNA, RNA or oligonucleotide
2. Specific activity and type of labelling system
3. Inherent sensitivity of detection system
4. Optimum hybridisation conditions
5. Target retention and accessibility
6. Degree of non-specific background inherent to the detection system.

Theoretical background

Complementary DNA strands are bound by relatively weak, non-covalent bonds including hydrogen bonds between guanine and cytosine (GC) and adenine and thymine (AT) base pairs; given sufficient energy these bonds break, denaturing the duplex.

The temperature at which half of a population of DNA duplexes denatures can be described by the value of T_m(°C) (Thomas & Dancis, 1973). This temperature varies depending upon several physical characteristics of the

nucleic acid strands involved and is a crucial factor in the determination of optimal hybridisation conditions.

T_m is calculated from the equation:

$T_m = 81.5°C + 16.61 \log M + 0.41(\%GC) - 820/L - 0.6(\%F) - 1.4\%$ (Mis), where: M = ionic strength (M/L), %GC = % Guanine/cytosine, L = probe length (bp), %F = %formamide (duplex stabiliser), % Mis = degree of non-complementarity

Increasing ionic strength stabilises duplexes and reduces the T_m of a particular duplex. The proportional GC content affects hybrid stability because GC base pairs are joined by three hydrogen bonds rather than two joining AT pairs. Clearly, long complementary pieces of duplex contain more bonds than short ones and are more stable. RNA–RNA duplexes are 10 to 15°C more stable than DNA–DNA duplexes of similar length and composition (Cox *et al.*, 1984) whilst DNA–RNA duplexes have intermediate T_ms (Casey & Davidson, 1977). As T_m is also dependent on probe length, it follows that lower hybridisation temperatures are needed for oligonucleotide probes. Hydrogen bonds between complementary nucleotides, especially in DNA duplexes, can be disrupted by formamide thus destabilising the duplex and lowering the T_m. Clearly the effect on non-homologous duplexes is greater than on homologous strands. Formamide is added to hybridisation mixtures so that optimal stringencies are possible at temperatures less deleterious to tissue sections.

In practice, *in situ* hybridisations are carried out at about $T_m - 25°C$. Such low stringency produces maximum probe hybridisation but also allows some hybridisation to non-homologous sequences. These more weakly bound duplexes can be preferentially denatured by low salt post-hybridisation washes at temperatures nearer to T_m. Hybridisation times are often determined empirically; however, knowledge of hybridisation kinetics and the factors which affect them can be used as a preliminary guide.

The time needed for half a population of probe to hybridise to the target sequence may be described in terms of $t_{\frac{1}{2}}$ which is calculated by the equation:

$$t_{\frac{1}{2}}\,(s) = \frac{N \ln 2}{3.5 \times 10^5} \times L^{0.5} \times C$$

where N = sequence complexity, L = probe length, C = [probe] (M/L).

In solution this relationship suggests that most rapid hybridisation occurs with fairly long, simple probes at high concentration. However, problems of probe penetration through tissue mean that shorter probes, of the order of 100 bp give the most rapid hybridisation for *in situ* hybridisation. In addition, penetration of probes may be further compromised by labels attached to the probe. This is due to steric hindrance and increased rigidity which can alter the shape, charge density and distribution of the probe.

Probe production

A detailed description of the microbiological procedures employed for the production of the various types of probe is outside the terms of reference for this chapter and can be seen in Maniatis, Fritsch & Sambrook (1982). However, an appreciation of the processes involved is important for the end user.

In most instances you will wish to localise previously characterised DNA or RNA sequences. The traffic in gene probes is very free at present but more often than not a donor laboratory will send only a small quantity (μg) of recombinant plasmid DNA or riboprobe vector in ethanol or buffer, or possibly as an agar stab preparation of recombinant *Escherichia coli*. Such quantities of DNA are enough for only a few *in situ* hybridisations and so it is necessary to amplify the amount of probe for future use.

Until very recently the methods of preparation, purification and labelling of sufficient quantities of pure probe have involved time-consuming, expensive and, very often, infuriating microbiological procedures which are beyond the logistic capabilities of many laboratories. Outlined below are some of the various steps involved in DNA production by standard molecular methods, but this is by no means exhaustive.

1. Quality control electrophoresis
2. Bacterial transformation.
3. Small-scale bacterial culture
4. Quality control electrophoresis
5. Large-scale bacterial culture
6. Plasmid purification
7. Restriction digest
8. Quality control electrophoresis
9. Preparatory electrophoresis
10. Band elution
11. Probe labelling (e.g. nick translation).

Even in experienced hands this process can take a couple of weeks.

The Polymerase Chain Reaction (PCR) (Saiki *et al.*, 1986) has altered the picture to the potential benefit of the end user. PCR can, under optimal conditions, produce micrograms of vector-free probe from a nanogram or less of DNA template. Recently PCR was used for direct production of biotinylated DNA probes (Lo, Mehal & Fleming, 1988) though the polymerase reaction efficiency is slightly reduced. Insert amplification depends on the use of specifically synthesised oligonucleotide primers complementary to sequences on alternate strands and at either end of the cloning site of the plasmid vector (Fig. 1). In the presence of an excess of

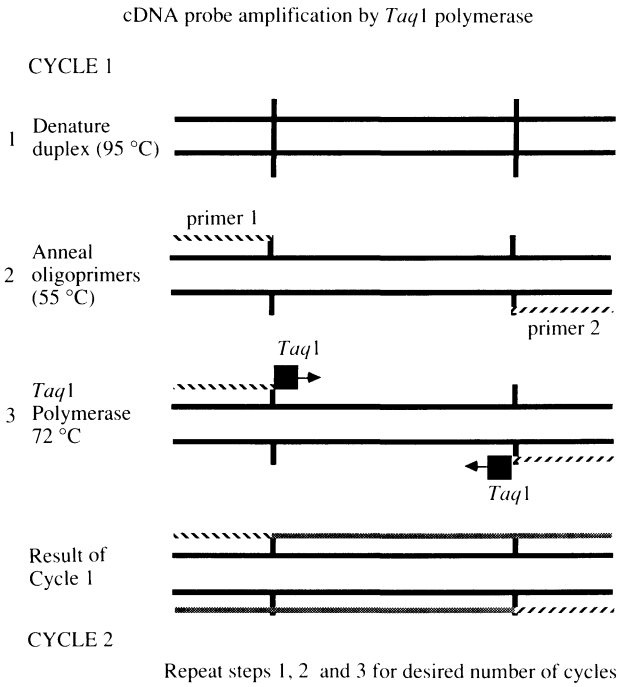

Figure 1. Schematic representation of the Polymerase Chain Reaction (PCR) for the amplification of cDNA inserts within recombinant plasmids.

nucleotides, from the bacterium *Thermus aquaticus* (Taq 1) polymerase will make a copy of the insert in the first cycle. This doubles the insert seed strands for the next polymerase cycle and so on. The geometric increase in insert copies soon produces the required amount of pure probe. Less than 1 ng of template DNA should be used in order to prevent rapid depletion of dNTP substrates. This method is successful for insert sequences of up to 2.5 kb. If PCR fulfils our expectations, it will make the histochemist independent by removing our reliance, however agreeable, upon molecular biologists.

Probe labelling methods

DNA probes

Two main principles underlie most of the methods for probe visualisation. Firstly, labelled nucleotides are enzymatically incorporated into probes during synthesis or afterwards by, for example, nick translation and visualised by standard immunocytochemical protocols. Secondly, probes may be chemically modified and visualised by binding to an immunogenic hapten and indirect antibody staining.

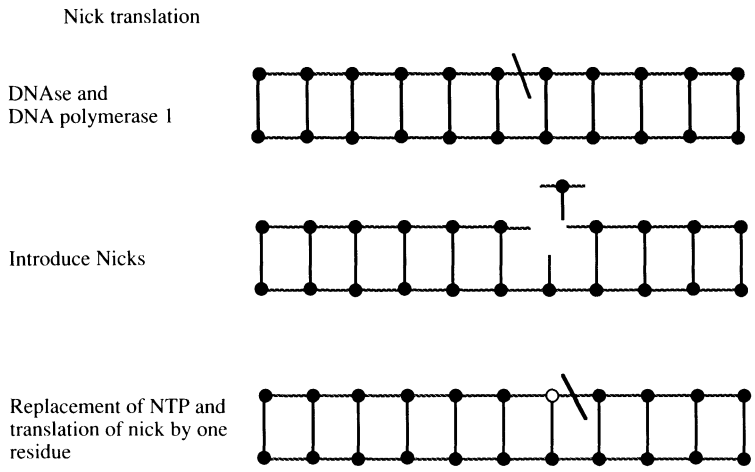

Figure 2. Schematic representation of the reactions involved in labelling of DNA by Nick translation using DNAse and DNA polymerase 1.

Nick translation (double-stranded DNA), Rigby *et al.*, 1977)

DNAse 1 makes cuts in one strand (nicks) at random sites on template DNA and DNA polymerase, via its 5′--3′ exonuclease and 5′–3′ polymerase activities, fills in the nicks with free deoxynucleotidephosphates (dNTPs) (Fig. 2). Labelled dNTPs are incorporated by this activity and, because the nicks are randomly sited, denaturation of the duplex produces strands of differing lengths. The specific activity of labelled probes depends on the extent of replacement by labelled nucleotides. Labelling can take between 30 minutes and 3 hours but long incubations cause degradation of labelled DNA. Gene-specific insert sequences may be purified and labelled or, alternatively, intact recombinant plasmid may be nick translated. This does not affect specificity and actually increases signal strength due to 'networking' of junction pieces (Lawrence & Singer, 1985).

Advantages

Control over probe size, yield, specific activity, substrate concentration and reaction time
60–70% incorporation of label
Standard hybridisation temperatures can be used
Large yield even of biotinylated probes
Labels circular and linear DNA
Uniform labelling.

Disadvantages
Unpredictable with impure template DNA
Uniform labelling not restricted to insert DNA
Needs large amounts of substrate
Requires careful control of time and temperature
Probe is not strand specific. Strand reannealing of probe may occur
Will not label single-stranded DNA.

Oligonucleotide primer extension synthesis

Random or unique oligonucleotide primers can be used with Klenow polymerase to synthesise new DNA strands complementary to a template strand, starting at the free 3′-hydroxyl terminus of the primer. It is essential to use a polymerase with no 5′–3′ exonuclease activity otherwise the primers will be degraded (Feinberg & Vogelstein, 1983, 1984).

Advantages
High specific activity
Efficient utilisation of label
Flexible reaction temperature and time (up to overnight)
Can label small amounts of DNA
Will label single stranded DNA
Incorporated label not excised during reaction
Probe size is uniform and controllable.

Disadvantages
Average yield only about 70 ng (i.e. only one hybridisation)
Relatively inefficient with circular DNA substrates
Labelling not limited to insert sequence.

Photobiotin labelling

Biotin is linked via a spacer molecule to a terminal aryl azide, which when activated to a highly reactive aryl nitrene by strong light, covalently links to the nucleotides in DNA or RNA (Fig. 3) (Forster *et al.*, 1985).

Advantages
Simple protocol
No enzymes involved.

Disadvantages
No real control over rate of incorporation (usually about 1 in every 100 nucleotides).

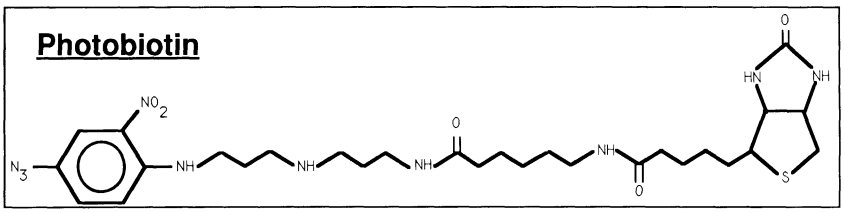

Figure 3. The molecular structure of photobiotin. Biotin is bound to a aryl azide group via a long spacer arm to reduce steric hindrance. Strong light converts the aryl azide to highly reactive aryl nitrene which binds to cytosine moieties in nucleic acids.

Labelling of single stranded DNA

Single-stranded DNA probes can be made *in vitro* using the M13 bacteriophage vectors. Potentially these probes are superior to double-stranded probes because they cannot reanneal. So far, limited use has been made of these probes owing to the difficulty of vector construction and the vagaries of probe synthesis and labelling (Varndell *et al.*, 1984).

Oligonucleotides

Increasingly, oligonucleotides are used for *in situ* hybridisations (for a review see Lewis, Sherman & Watson, 1985). They can be simply and reliably synthesised using standard phosphotriester or phosphoramidite chemistry given the availability of an automatic synthesiser. Oligonucleotides have a number of advantages over cloned DNA probes including:

Higher and controllable specific activities
Amino acid sequences can act as blueprints in the absence of DNA sequence data
Multiple probes can be generated for a single sequence. Thus specificity can be tested by signal strength and cellular distribution
Sense probes can be used as very specific negative controls.

Oligonucleotides may be 5′ end-labelled by T4 polynucleotide kinase (Richardson, 1981) or 3′ end-labelled by terminal deoxynucleotide transferase activity (Bollum, 1974; Maniatis *et al.*, 1982). High specific activity can be achieved by end-labelling with biotinylated dNTPs. Densely biotinylated oligonucleotides suffer from steric hindrance reducing hybridisation efficiency.

RNA probes

Detailed descriptions of the production and use of riboprobes have been given elsewhere (e.g. Angerer *et al.*, 1987 and in Chapters 3 & 4). Only

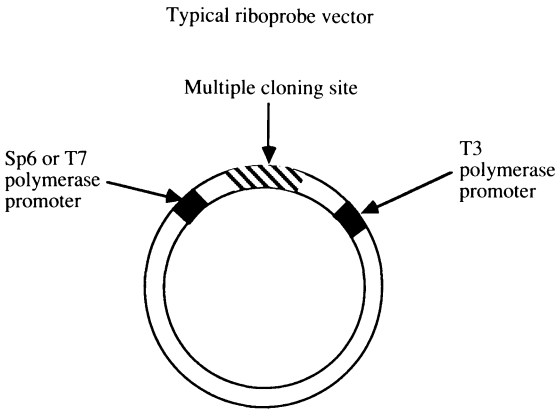

Figure 4. Diagram of the organisation of a typical riboprobe showing the orientation of Sp6 and T7 polymerase promoter sequences.

those features pertinent to the central theme of this essay will be described here. Single-stranded RNA probes can be made *in vitro* from RNA transcription vectors which contain promoter sequences for RNA polymerases such as SP6, T3 and T7 (Cox *et al.*, 1984) (Fig. 4). Antisense riboprobes are far more sensitive with lower background than double-stranded DNA probes because they do not suffer from probe reannealing. Other advantages of riboprobes over cDNAs are that they can be labelled to higher specific activity, they are thermally stable and have constant and defined probe size, all of which favours increased sensitivity and consistency of results. However, so far there are relatively few reports of the use of non-radioactively labelled RNA probes. This may be because biotinylated nucleotides are poor substrates for RNA polymerases (Höfler, 1987). A comparison has been made between biotinylated and [^{35}S] labelled riboprobes complementary to portions of human papilloma virus-16 RNA for use in *in situ* hybridisation (Crum *et al.*, 1988). Biotinylated riboprobes were transcribed in GEM 1 vectors with either T7 or Sp6 polymerase. Sp6 transcription was also used by Zabel & Schäfer (1988) to make biotinylated riboprobes to localise calcitonin and calcitonin-gene related peptide mRNAs in rat parafollicular cells. Neither of these studies reported problems with biotin incorporation but Crum and co-authors indicated lower sensitivity of the biotin labelled riboprobe Allyl-UTP and subsequent biotinylation has been tried with limited success due to high background problems (Höfler, 1987). Photobiotin methods are also being adapted for RNA probes, and some of the chemical modification methods such as sulphonation or mercuration may prove useful in releasing the true potential of this powerful system.

Non-radioactive labelling systems

Despite the power of *in situ* hybridisation using radiolabelled probes there are serious limitations. Many isotopes, for example ^{32}P, have short functional half-lives, are expensive, and require stringent safety controls for workers and disposal. The development of alternative sensitive, safe and rapid label systems has been highly desirable (for a review see van der Ploeg *et al.*, 1986). The main advantages and disadvantages of non-radioisotopic labels are listed below.

Advantages	
Probe stability	Signal amplification
Safety (handling and disposal)	Inexpensive
Rapid detection	High resolution (E.M.)
No specialist labs	Quantifiable (in certain cases)
Multiple, simultaneous labelling	Compatible with immunohistochemistry.

Disadvantages	
Decreased hybridisation efficiency.	Claimed low sensitivity.

Labelling systems currently used for *in situ* hybridisation include:

Biotin–avidin (streptavidin) conjugates, Biotin–anti-biotin conjugates, Photobiotin–avidin conjugates, Sulphonated nucleotide–antibody conjugates, Mercurated nucleotide–antibody conjugates, Acetylaminofluorenyl–antibody conjugates, Bromodeoxyuridine–antibody conjugates, Fluorochrome-labelled nucleic acids and anti-DNA:RNA hybrid antibodies.

Biotin–avidin labelling systems are the most commonly used non-radioisotopic technique. Localisation of hybridised probe depends upon the high binding constant of biotin (Vitamin H derived from egg white) for avidin (glycoprotein from egg yolk). The association constant (10^{15} M^{-1}) is some 10^6 times greater than typical antigen–antibody affinities. Biotin can be enzymatically attached to the C-5 position of pyrimidine rings of nucleic acids via an allylamine linker arm (Langer, Waldrop & Ward, 1981) but high-quality biotinylated nucleotide phosphates can be obtained commercially (Enzo or Sigma) for use in labelling reactions. DNA polymerases will readily use biotinylated bases as substrates but RNA polymerases utilise biotinylated nucleotides less efficiently. Biotin is usually bound to the probe via a long spacer molecule (e.g. Biotin 11-UTP), in order to reduce steric hindrance and increase hybridisation efficiency. Biotinylated probes