CHAPTER 12

In Situ Hybridization

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I. Introduction
II. Which Protocol to Use?
   A. Preparing Wax Sections of Plant Tissue
III. Labels, Tags, and Detection Systems
   A. Isotopic Labels
   B. Nonisotopic Labels
IV. Probe Types
   A. Oligonucleotides
   B. Double-Stranded DNA Probes
   C. Single-Stranded RNA Probes
   D. Making "S-Labeled RNA Probes
V. Hybridization Conditions
   A. Hybridization Protocol
   B. Autoradiography
VI. Controls
VII. New Techniques
    References

I. Introduction

In situ hybridization is a cytochemical technique for localizing specific DNA or RNA sequences within an organism. Like Northern and Southern blots, in situ hybridization relies on Watson/Crick base pairing (commonly termed hybridization) between two polynucleotides with complementary sequences. However, unlike Northern and Southern blots, where the nucleic acids are first isolated and then separated by electrophoresis prior to probing, for in situ hybridization...
the target sequences are left within the tissue. The probe, which is labeled, is sent into the tissue, allowed to hybridize with complementary target sequences, then rendered visible with some form of marker. Applied correctly the in situ hybridization technique provides precise information about the location of a specific DNA or RNA sequence. It is an extremely powerful tool for studying tissue-specific expression, and is also becoming an invaluable adjunct in chromosome mapping.

Applications of in situ hybridization technology are diverse. The most common usage in plant cell biology is localization of a mRNA at the light microscopic level, and this chapter will focus on methods for this. Exciting new applications, such as localization of DNA elements on isolated chromosomes (Heslop-Harrison et al., 1993), localization of genes or transcripts at the subcellular level by electron microscopy (McFadden, 1991), and in situ PCR (Nuovo, 1992) are also introduced briefly.

II. Which Protocol to Use?

Because of the wide range of applications, there is unfortunately no single universal protocol for localizing nucleic acid species. Each worker will have to adopt a protocol suited to their system. This depends on what type of tissue they are working with, and what level of resolution they require. Various claims are made about the relative sensitivity of in situ hybridization, but in my experience only those mRNAs readily detected by Northern analysis can be successfully localized by in situ hybridization. However, it can occur that a mRNA that is highly localized within a particular cell type is easily detected by in situ hybridization but is difficult to detect by Northern blot analysis. This is due to the dilution effect of nonexpressing cells included in the tissue sample from which the RNA is extracted. Conversely, a mRNA present in low levels throughout all cell types may be readily detected by Northern analysis, but could be difficult to detect by in situ hybridization.

A problem somewhat peculiar to in situ hybridization analyses using plant tissues is the presence of vacuoles and cells of differing size. These features result in very different amounts of cytoplasm per unit section area in different tissues, and this should be taken into account when interpreting the relative levels of signal in a section. Tissues with small vacuoles in the cells (such as the tapetum, vascular bundles or apical meristems) will be most heavily labeled, even though the gene may not be most highly expressed in these tissues. A useful way to test this is to use a rRNA probe as a positive control to get an overview of cytoplasmic density in comparison with expression of the gene of interest (e.g., Fig. 5 in McFadden et al., 1991).

Greatest success in localizing mRNAs in plant tissues has been achieved using 3S-labeled RNA probes on wax sections. This approach was pioneered by Angerer and Angerer (1989) and adapted to plant systems by Cox and Goldberg.
Improved protocols for \(^{35}\text{S}\)-labeled RNA probes on wax sections are presented in detail here. Protocols for nonisotopic labeling and production of resin or cryosections can be found elsewhere (McFadden, 1994; Boehringer Mannheim, 1992; Brunning et al., 1993).

A. Preparing Wax Sections of Plant Tissue

Embedding tissue in wax requires fixation and dehydration prior to infiltration with the embedding medium. Aldehyde fixation is optimal, and a combination of 4% paraformaldehyde and 0.5% glutaraldehyde suits most plant material. The fixative should be prepared fresh in a buffer isotonic to the growing conditions. Do not use Tris in conjunction with aldehyde fixatives; phosphate or "Good" buffers such as Hepes or Pipes are best. Fixation of plant tissues with extensive waxy cuticles can be improved by use of the phase-partition fixation technique (McFadden et al., 1988a, and see section II,A,3).

1. Solutions and Materials

- Plastic slide staining boxes holding 25 slides from Vit-Labs/Vitri Friedrich-Ebert Str 33-35, D-6104 Seeheim-Jugenheim, Germany
  1% (v/v) solution of Decon 90 detergent (Rhone Poulenc)
  poly-L-lysine (0.1 mg/ml)
  distilled water
  100 mM Pipes (pH 7.3)
  20% formaldehyde (Ladd Research Industries)
  8% glutaraldehyde (Serva GmbH, Carl Benz Str 7, D-6900 Heidelberg, Germany)
  10% ethanol, 20% ethanol, 30% ethanol, 40% ethanol, 50% ethanol, 60% ethanol, 70% ethanol, 90% ethanol, 100% ethanol
  4% paraformaldehyde (v/v), 0.5% glutaraldehyde (v/v) in 50 mM Pipes (pH 7.3)
  n- heptane
  Clearene (less-toxic alternative to xylene, Surgipath Medical Industries, Inc., P.O. Box 769, Ziegler Drive, Graylake, IL 60030)
  Paraplast embedding wax chips (BDH Chemicals Ltd., Poole BH12 4NN, UK)
  embedding molds (Tissue Tek, No. 4566, Miles, Inc., Elkhart, IN 46515)
  pronase (0.1 mg/ml in Tris–HCl, pH 7.6)
  glycine/histidine (2 mg/ml each in PBS)

2. Coating Slides

Fit slides into rack of Vitri box and immerse in detergent for 5 min. Rinse slides well with water and air dry. Dip slides in poly-L-lysine for 5 min. Drain and air dry away from dust.
3. Fixing Tissue

Prepare fresh paraformaldehyde (4%) and glutaraldehyde (0.5%) in 50 mM Pipes (pH 7.3). Combine fixative with equal volume of n-heptane and shake for 1 min. Allow phases to separate, then decant heptane (upper) phase. Retain aqueous phase for subsequent step. Immerse small pieces of tissue (about 2 × 2 × 2 mm) in fixative-loaded heptane for 10 min at room temperature. Transfer tissue pieces to aqueous paraformaldehyde (4%) and glutaraldehyde (0.5%) in 50 mM Pipes (pH 7.3) and fix for further 2 h at room temperature. Wash in buffer three times and proceed to wax embedding.

4. Wax Embedding

Dehydrate fixed tissue through ethanol series (10, 20, 30, 40, 50, 60, 70, 90, 100%) allowing at least 15 min for each step. Transfer tissue into a glass tube containing a mixture of 3 parts ethanol and 1 part Clearene for 60 min. Transfer tissue to a mixture of 1 part ethanol and 1 part Clearene for 60 min. Transfer tissue to a mixture of 1 part ethanol and 3 parts Clearene for 60 min. Transfer tissue to straight Clearene for 60 min. Add several chips of Paraplast embedding wax to each sample in Clearene and leave overnight at room temperature. Add several more chips of Paraplast and transfer tube to 42°C waterbath for 3 h. Place several Paraplast chips in an embedding mold and melt wax in oven at 60°C. Using warmed forceps, transfer tissue pieces from tubes to embedding molds. Incubate for 24 h at 62°C. Remove and store at room temperature.

5. Preparing Wax Sections

Cut sections of desired thickness (3–6 μm) on suitable microtome. Transfer sections to droplet of water on poly-L-lysine-coated slides. Dry down on 42°C hotplate for 2 h. Only perfectly flat sections should be retained. Using a diamond pencil, scratch a circle around the section on the back of the slide. Dewax sections by soaking slides in Clearene for 10 min followed by two washes in ethanol for 5 min each. Rehydrate sections by transferring through graded ethanol series (95, 80, 60, 30%) allowing at least 5 min in each step. Transfer to distilled water. Air dry and store at 4°C. Digest sections with pronase (0.1 mg/ml in Tris–HCl, pH 7.6) for 10 min at room temperature. The amount of digestion may need to be varied with different tissue types. Monitor nucleic acid retention by acridine orange staining (McFadden et al., 1988b). Deactivate pronase by dunking slides in glycine/histidine (2 mg/ml in PBS). Wash twice in water and air dry.

III. Labels, Tags, and Detection Systems

There are two major categories of labels and detection systems for in situ hybridization: isotopic and nonisotopic. Both have relative merits, depending on
the system used. Isotopic labeling is familiar to most molecular biologists, and standard probe production techniques (nick-translation or random priming) can be used. However, microautoradiography (the method used to visualize the bound probe) is an exacting procedure, and not always easily mastered. Nonisotopic systems are safer, have potentially higher resolution and sensitivity, and produce probes with longevity.

A. Isotopic Labels

Isotopic labels are detected by autoradiography. Latent silver grains produced by β particles colliding with silver halide in an emulsion are chemically developed (Pardue, 1986). Macroscopic autoradiography can be performed by apposing fine-grain, single-coated X-ray film directly to the labeled sections. Microautoradiography involves coating the sections with a very thin layer of melted emulsion. After a suitable exposure period, silver grains in the emulsion are chemically developed.

Three isotopes emitting β particles commonly used for in situ hybridization are 32P, 35S, and 3H. The β particles from 32P have the highest energy and spread of the particles results in low resolution. However, 32P has the highest specific activity and this isotope gives maximum sensitivity in low-resolution systems (Fig. 1). Although seldom used, 32P is potentially well suited to in situ hybridization applications because it combines a high specific activity with a relatively low β particle energy (Evans and Read, 1992). 35S β particles have a lower emission energy and provide better resolution. The longer half-life of 35S extends probe longevity slightly. 35S-labeling is most useful for applications localizing mRNAs to tissues or cell layers in sections (Fig. 2). Very fine resolution is achieved with 3H, which has the lowest emission energy of the three. Subcellular localization can be achieved using 3H-labeled probes for electron microscopy (Penschow et al., 1991). The specific activity of 3H-labeled probes is relatively low and prolonged exposures can be necessary, but the probes do have the advantage of being extremely long-lived.

B. Nonisotopic Labels

Various nonisotopic labels or tags are now in use, and they can be separated into two categories: direct and indirect. Direct refers to labels that are themselves intrinsically visible. Indirect refers to labels that must be rendered visible by subsequent manipulation. The most common direct labels are fluorochromes. Fluorochromes emit visible light when excited by UV radiation in the light microscope. Direct fluorochrome labeling is relatively simple (Boehringer Mannheim, 1992; Brunning et al., 1993), and the bound probe is immediately detectable by fluorescence microscopy after post-hybridization washing.

Indirect methods involve initial incorporation of a tag molecule (the label) into the probe. Tags on the bound probe are then detected with an antibody or
affinity reagent to which is coupled a visible marker (such as a fluorochrome or a gold bead) or an enzyme (such as alkaline phosphatase), which can be supplied with a substrate that it converts to an insoluble, visible product. Indirect methods are more complex than direct methods, but they offer greater flexibility in detection methods and the potential to amplify signals (McFadden, 1994).

Nonisotopic labeling is best done by enzymatic incorporation of tagged nucleotides into a probe molecule produced from a cloned template (Langer et al., 1981). The tag or label is usually attached at the C5 position on a pyrimidine. The C5 position is not involved in base pairing during hybridization, and modifications do not adversely affect recognition of the nucleotide substrate by commonly used nucleic acid modifying enzymes. The tag is attached to the base by an allylamine spacer arm that keeps the tag accessible to the detection reagents, even though the probe is duplexed with the target. An 11- or 14-atom spacer arm is most effective. Labeling of RNA probes is normally done by incorporating tagged rUTP. Oligos and double-stranded DNA probes are labeled using tagged dUTP as a dTTP analogue.

Biotin (vitamin H) was the first tag employed for in situ hybridization. It was originally chosen because of the extraordinary affinity of the egg white protein avidin for biotin (Wilchek and Bayer, 1989). Avidin and also the bacterial cell wall protein streptavidin bind to biotin with dissociation constants in the order of $K_{d}$, $10^{-15}$. When these proteins are coupled to indicator molecules, an excellent affinity detection system can be devised.

Virtually any moiety can be used as a tag, providing a suitable means for detecting the tag is available and that the tag's presence does not disrupt hybridization. Digoxigenin, the aglycone of the plant cardiac glycoside digoxin, has recently been introduced as an alternative to biotin, which was sometimes found to be unsuitable as a tag in animal tissues with high levels of endogenous biotin. Digoxigenin (DIG) is incorporated into probes in the same ways as biotin (Boehringer Mannheim, 1992). Detection is via an anti-digoxigenin antibody. The only other tag commonly used for in situ hybridization is dinitrophenol (Narayanswami and Hamkalo, 1991).

IV. Probe Types

Three types of probes can be used for in situ hybridization: synthetic oligodeoxyribonucleotides (oligos), double-stranded DNA probes (i.e., cDNAs or genomic clones), and single-stranded RNA probes (i.e., antisense/sense RNAs, cRNAs, or "riboprobes"). Each of these probe types has advantages and disadvantages.

A. Oligonucleotides

Oligonucleotides (which are usually between 15 and 50 nucleotides in length) are now readily available to most workers and can be produced relatively quickly.
Working with oligos requires no cloning expertise and probes can be produced by any worker from published sequence data. A notable advantage of oligos is that they can be directed to particular portions of the target species. Several different oligos directed at the coding region, introns, or untranslated regions can be used to localize the same target and provide information about target processing in different tissues.

Various labeling and detection strategies are possible with oligos. Isotopic labeling can be achieved by kinasing with T4 polynucleotide kinase using $\gamma^{[32P]}$NTP, $\gamma^{[3H]}$NTP, or $\gamma^{[35S]}$NTP (McFadden, 1994) or by tailing with terminal transferase to add tagged dNTPs at the 3' end (Boehringer Mannheim, 1992).

Nonenzymatic attachment of tags or labels to oligos is also possible using the "amino-link" system (Applied Biosystems User Bulletin No. 49, 1988). An amine group is incorporated at the 5' end of the oligo during synthesis. When the amine-bearing oligo is mixed with an N-hydroxysuccinimide ester of a fluorochrome, biotin, or DIG at alkaline pH, the unprotonated amine attacks the ester linkage in the NHS-tag complex, resulting in an amide bond between the oligo and the tag molecule. The NHS tag esters have allylamine linker arms.

Recently, biotin (Misiura et al., 1990) or fluorescein amides (Applied Biosystems Product Bulletin, 1993) have become available, allowing biotin or fluorescein to be incorporated directly into the oligonucleotide during synthesis. The amides are usually incorporated at the 5' prime end during the last synthesis cycle but can be incorporated at any chosen site in the oligonucleotide.

B. Double-Stranded DNA Probes

These probes are produced from cDNA or genomic clones that have been amplified in a cloning vector supported by a suitable bacterial host (Ausubel et al., 1988). Double-stranded probes can be labeled with isotopes or nonisotopic labels by either of two methods: nick-translation or, preferably, random priming (Feinberg and Vogelstein, 1983; McFadden, 1994). Double-stranded refers to the fact that both the coding strand and the noncoding strand are included in the probe. Although they are reasonably satisfactory for localization of DNA, double-stranded probes suffer from some major disadvantages when localizing RNA targets. Only the coding strand hybridizes with RNA targets. The labeled noncoding strand is not only superfluous, it actually hybridizes back to the coding strand, thereby competing against the target. Moreover, because the unlabeled template remains with the probe, it competes with the labeled probe to hybridize with targets, severely reducing the signal. Additionally, the low yield of probe produced (typically less than 100 ng) is only sufficient for a small number of in situ hybridization experiments.

C. Single-Stranded RNA Probes

RNA probes complementary to cellular RNA can be produced by in vitro transcription. The antisense RNA of cRNA (complementary RNA) is produced
from a vector in which the noncoding strand is sited downstream of a bacterio-
phage RNA polymerase promoter. Discrete length "run off" transcripts incorpo-
rating either isotopically or nonisotopically labeled nucleotides are made from
a linearized plasmid template using phage RNA polymerase. Large quantities
of probe can be produced in this manner. The unlabeled DNA template is rela-
tively insignificant as a competitor to the probe but can be removed by DNase
digestion. Another advantage of RNA probes is the stronger hybrids formed
between two strands of RNA, allowing a higher stringency (see Section V).

1. False Binding with RNA Probes

A potentially serious drawback with RNA probes is a tendency to bind nonspe-
cifically to rRNAs. Recently, it was demonstrated that very short G/C-rich motifs
in riboprobes can hybridize to complementary sequences in the rRNAs of human
tissue (Witkiewicz et al., 1993). Several 9-base pair motifs comprising the NorI,
SmaI, and SacI sites in the poly linker of the transcription vector pBluescript KS
II (Stratagene, Inc.) were found to have complements in the human large subunit
rRNA (Witkiewicz et al., 1993). Somewhat surprisingly, these 9-base pair motifs
cross-hybridize with rRNA at standard stringency (50% formamide, 1× SSC,
50°C), probably because RNA/RNA duplexes are particularly stable. (Witkiewicz
et al., 1993). Transcripts of the poly linker without insert gave the same signal
as those with the insert, and Northern blotting revealed that the majority of
hybridization was to the large subunit rRNA (Witkiewicz et al., 1993).

I have examined the potential for similar problems in plant systems and found
that commonly employed transcription vectors (pBluescript, pBluescript II, and
several of the pGEM series) contain motifs with complements in a number of
different plant rRNAs. For instance, the T7 transcript through the SmaI site
of pBluescript SK (5'CCCCCGGGGCT3') has a complement in the large sub-
unit rRNA of Oryza sativa, and the T3 transcript through the NorI site of
the same vector (5'GCGGCGGCCGCC3') also has a complement in the same rice
rRNA. Similarly, the T7 transcript through the ApaI site of pBluescript KS
(5'CGGCCCCCCCCCC3') has a complement in the rice chloroplast large subunit
rRNA. These and other motifs represent a possible source of spurious hybridiza-
tion, and serious consideration should be given to the design of an in situ hybrid-
ization experiment employing RNA probes. Depending on the site and orienta-
tion of the insert within the transcription vector, either the sense or the antisense
RNA probe strand could cross-hybridize with plant rRNA.

There are a number of precautions that should be followed to avoid being
misled by such spurious hybridization. Best practice would be to perform a
Northern blot with the same RNA probe to determine that the probe is actually
detecting your mRNA and not a rRNA. This blot should be done at an equivalent
or slightly lower stringency (see Section V) than the in situ hybridization. Another
useful control would be to run a parallel in situ hybridization using run off tran-
scripts of the same poly linker without any insert to check for spurious signal in
either the sense or the antisense. Where practical it would be prudent to eliminate G/C-rich motifs from your in vitro transcript system by linearizing the vector upstream of these regions. Some of the pGEM series (e.g., pGEM3z, pGEM3zf, pGEM4z, and pGEM7zf, Promega Corp.) lack G/C-rich motifs in their polylinkers, and it might be worthwhile using these vectors where possible, but there is no guarantee that these polylinkers, or even your own insert, do not contain other motifs that could cross-hybridize to nontarget RNAs in your tissue. Good controls will be essential in establishing a bona fide signal. It should also be possible to minimize problems by using higher stringency. I routinely perform hybridization with short RNA probes (ca. 100 bp) at 65°C in 50% formamide, and up to 70°C for longer probes (800–2000 bp). The sense strand should no longer be considered an adequate control, because it is possible to have a false positive with the antisense but still get a satisfactory negative control with the sense strand because it does not contain the same motif.

D. Making ³²P-Labeled RNA Probes

This method produces single-stranded RNA probes complementary to the target. The cloned sequence must be subcloned into an in vitro transcription vector such that the noncoding strand is downstream of a bacteriophage RNA polymerase promoter engineered into the vector. The polymerase then transcribes a complementary or antisense RNA from the template. Most commercially available vectors have a different RNA polymerase promoter on either side of the multiple cloning site so that the sense RNA can be transcribed from the same construct simply by using a different polymerase. By cutting the vector with a restriction endonuclease downstream of the insert, it is possible to limit the transcription to discrete length runoffs and produce labeled probe molecules of defined length. The pBluescript II vectors (Stratagene, La Jolla, CA) have restriction sites for BssHII flanking the RNA polymerase promoters allowing a transcription cassette, suitable for both sense and antisense transcriptions, to be released with a single enzyme digestion.

A typical in vitro transcription will produce several micrograms of labeled RNA probe. RNA is easily destroyed by ribonucleases. These enzymes are extremely resilient and are easily transferred from our skin to reaction tubes and pipette tips. Always wear gloves while preparing RNA probes, autoclave all tubes and tips, and treat water for all buffers with DEPC (Ausubel et al., 1988).

1. Materials and Reagents

- template DNA
- 5X transcription buffer (200 mM TRIS, pH 7.5, 30 mM MgCl₂, 10 mM spermidine, 50 mM NaCl)
- 100 mM DTT
RNasin (20 U/μl, Promega Corp, 2800 Woods Hollow Rd, Madison, WI 35711-5399)
10 mM ATP, 10 mM GTP, 10 mM UTP
phage RNA polymerase (20 U)
DEPC-treated water
RQ1 RNase-free DNase (1 U/μl, Promega)
phenol/chloroform saturated with TE
chloroform : isoamyl alcohol (24 : 1)
BioSpin 30 column (Bio-Rad, 3300 Regatta Blvd, Richmond CA 94804)
1 liter TBE (0.1 M Tris-borate, 2 mM EDTA, 0.1 M boric acid)
agarose
1% ethidium bromide (w/v)
loading dye [0.25% (w/v) bromphenol blue, 40% (v/v) glycerol]
agarose gel electrophoresis unit
distilled water
RNA ladder (Bethesda Research Labs/GIBCO, MD)
0.2 M Na₂CO₃
0.2 M NaHCO₃
3 M NaOAc (pH 6)
10% glacial acetic acid
4 M LiCl
ethanol
hybridization buffer (50 mM piperazine ethane sulfonic acid (pH 7.2), 0.15 M NaCl, 5 mM Na₂-ethylenediaminetetraacetic acid (EDTA), 100 μg ml⁻¹ powdered herring sperm DNA (Type D-3159, Sigma), 0.1% Ficoll 400 (Pharmacia, Sweden), 0.1% polyvinyl pyrrolidine 40, 0.1% BSA, 50% deionized formamide. Aliquots of buffer should be stored frozen.

2. In Vitro Transcription

Linearize template and determine concentration (Ausubel et al., 1988). Avoid using enzymes that generate 3’ overhangs, as these allow the polymerase to turn back and continue transcribing off the other strand producing a hairpin probe. If an enzyme generating 3' overhangs must be used to linearize, blunt the ends with T4 polymerase (Ausubel et al., 1988).

At room temperature add these reagents in this order: 4 μl of 5X transcription buffer, 2 μl of 100 mM DTT, 1 μl of RNasin, 1 μl of 10 mM ATP, 1 μl of 10 mM GTP, 1 μl of 10 mM UTP, 5 μl of α[³²P]CTP (10 mCi/ml), 1 μl of template DNA (0.5–2.0 μg), 1 μl (20 units) of appropriate RNA polymerase (T7, T3, or SP6, depending on which promoter is upstream of the insert), sufficient DEPC-treated water to make a final reaction volume of 20 μl. Mix components. Incubate
at 37°C for 1 h. Remove a 3-μl aliquot and set aside for electrophoretic analysis (Section IV.D.3) if required.

3. Gel Electrophoresis of Labeled Probe

When preparing RNA probes it is advisable to check the transcripts by electrophoresis. A simple nondenaturing gel is adequate for this purpose. Dissolve 0.5 g of agarose in 50 ml of TBE in microwave oven. Add 2 μl of 1% (w/v) ethidium bromide (ethidium bromide is toxic). Pour agarose solution into “mini-gel” casting mold and insert comb (see Ausubel et al., 1988, for more detail). Allow gel to set before removing comb. Add 1 μl of loading dye and 7 μl of water to the 3 μl set aside from the transcription reaction. A useful standard is 3 μg of “RNA ladder” 9.5–0.24 knt. Add the dye and water as above. Load the samples and electrophorese at 5 V/cm for 1 h. View the gel using UV 300-mm transilluminator. The apparent molecular weight of the labeled transcripts will not be exactly correct due to the nondenaturing conditions. An approximate estimate of the yield can be made by comparing the fluorescent intensity of the transcript band with that of one of the bands in the RNA ladder standard. It is also possible (but not optimal) to run a gel using a portion of the probe after it has been precipitated and resuspended in hybridization buffer. The buffer and electrophoretic apparatus will need to be decontaminated for radioactivity.

4. Removal of Template and Unincorporated Nucleotides

Add 2 μl of RQ1 RNase-free DNase to transcription mix and incubate 15 min at 37°C. Add 180 μl of DEPC-treated water. Extract with phenol/chloroform followed by chloroform : isoamyl alcohol (24 : 1). Pass the aqueous phase through a spin column to remove unincorporated nucleotides by exclusion chromatography.

5. Probe Size

If the probe is less than 600 nt, I recommend using the full-length probe. Longer probes should be cut into shorter fragments by limited alkaline hydrolysis as follows. The optimal size is between 150 and 400 nt, depending on the porosity of the tissue. Estimate the volume of the spin column eluate. For each 100 μl of eluate add 60 μl of 0.2 M Na₂CO₃ and 40 μl of 0.2 M NaHCO₃. Calculate the appropriate hydrolysis time from the equation

\[ t = \frac{(L_o - L_f)\lambda}{(KL_oL_f)\lambda} \]

where \( L_o \) = length in knt of your runoff transcript, \( L_f \) = desired length of hydrolysis products, \( K = 0.11 \), and \( t \) = time in minutes. For instance, if the runoff transcript is 2 knt, and it is decided to cut the probe down to pieces with an average length of 0.5 knt, 14 min hydrolysis is required. Stop the hydrolysis by
adding 3 M NaOAc to a final concentration of approximately 0.1 M then add 10% glacial acetic acid to a final concentration of 0.5%. The size of the hydrolysis fragments can be checked by electrophoresis as described in Section IV.D.3.

6. Precipitation of Probe

Once the unincorporated nucleotides have been removed using the spin column and size reduction has been accomplished, it is necessary to precipitate the probe and resuspend it in hybridization buffer. Estimate the volume of the spin column eluate or the terminated hydrolysis mix if size reduction was undertaken. Add 0.1 vol of 4 M LiCl. Add 2.5 vol of ethanol. Stand in freezer for at least 1 h. Spin in microfuge at 4°C for 15 min. Pour off supernatant and stand tube upside down to drain for 15 min. Resuspend the pellet in suitable volume of hybridization buffer.

V. Hybridization Conditions

Hybridization of the probe to the target is governed by a range of physical parameters. The hybridization occurs through base pairing to create hydrogen bonds linking the probe to the target. The number of hydrogen bonds determines the strength of the hybrid. Shorter probes form less stable hybrids. Probes that do not have a perfectly complementary sequence (often known as heterologous probes) also form less stable hybrids. For a successful in situ hybridization experiment, conditions maximizing specific annealment of the probe to the target must be determined. The conditions under which hybridization is attempted are known as the "stringency," where low stringency allows weaker hybrids to remain as hybrids and high stringency is less permissive. The stability of the hybrid is represented by the melting temperature (Tm), the temperature at which half the population of hybrids become dissociated or "melted." A guide to stringency conditions can be obtained from the following relationship, which predicts Tm for two RNA molecules:

\[
T_m = 79.8°C + 18.5 \log M + 0.584(\%G/C) - 300 + 200M/L - 0.35(\%F) - 1.4(\%mismatch),
\]

where \( M = \) ionic strength (mol liter \(^{-1}\)), \( \%G/C = \) mole percentage of guanine/cytosine pairs in the probe/target hybrid; \( L = \) length of the probe in nucleotides;

\(^1\) The volume of buffer used to resuspend the probe is dependent on the amount of probe synthesized and the concentration at which the probe is to be used. Probe concentrations in the order of 1 μg/ml are usually suitable. It is better to maintain consistent probe concentration than to vary the amount of probe in order to apply equivalent counts in each experiment. Resuspend probe in a minimal volume and test a dilution series to establish which dilution produces the optimal signal-to-noise ratio in your system. Remember, excess probe will increase the nonspecific hybridization and create noise. Store probe in hybridization buffer at –20°C or use immediately.
%F = percentage (v/v) of formamide in the hybridization buffer; and %mismatch = the percentage of noncomplementary base pairs between the hybridizing strands. Thus, by increasing the salt concentration, or decreasing the formamidizing concentration, or decreasing the hybridization temperature, the stringency can be lowered, allowing short probes or probes with a small degree of sequence mismatch to be used. Conversely, higher stringency can be attained by increasing the formamide concentration, or lowering salt concentration or by increasing temperature. Formamide increases the solubility of the bases and is a duplex destabilizing agent; it is usually used at 50% concentration. This allows lower temperatures to be used, minimizing damage to the tissue sample. Standard hybridization buffer includes 50% formamide and 0.15 M NaCl, and it is usually best to keep this constant and adjust stringency by varying the temperature. The simplest way to optimize an in situ hybridization system is to run a series of experiments using the standard buffer at different temperatures and monitor the signal-to-noise ratio. As a guide, DNA/DNA hybridization should be done at 37–42°C, DNA/RNA hybrids at 42°C, and RNA/RNA hybrids at 50–70°C. When working with oligos, decrease the formamide concentration to 40%.

Posthybridization washes are done at a temperature equivalent to or higher than the hybridization temperature. Salt concentration can be reduced in the wash buffer to increase stringency, but best results are obtained by optimizing the hybridization conditions rather than trying to reduce noise with stringent posthybridization washing; once probe has annealed in situ it is hard to dislodge.

A. Hybridization Protocol

1. Materials

   hybridization buffer (Section IV,D,1)
   SSC—Prepare a stock of 20× SSC (3 M NaCl, 0.3 M Na citrate) and store at room temperature
   ethanol (technical grade)
   ethanol (AR grade)
   0.1 M triethanolamine–HCl, pH 8.
   acetic anhydride
   circular coverslips (12 mm diameter)
   chloroform
   probe in hybridization buffer
   plastic meat defrosting container with draining rack for hybridization box

2. Prehybridization

   Dip slides with sections in hybridization buffer at room temperature for 5 min. Transfer to hybridization buffer at 38–42°C for 1 h. Rinse in 1× SSC. Dehydrate
in ethanol (technical grade) followed by ethanol (AR grade). Drain and store up to 4 weeks at 4°C in a closed staining box containing about 4 ml of ethanol.

3. Acetylation of Slides

Slides coated with poly-L-lysine should be acetylated to prevent probe from adhering to the coating. Dip slides in 0.1 M triethanolamine–HCl, pH 8. While stirring vigorously, add acetic anhydride to concentration of 5 ml/liter. Wash slides in 2× SSC for 5 min. Drain and air dry.

4. Hybridization and Washing

Wash circular coverslips in chloroform and dry. Place coverslips on small, dark platform (e.g., the lid of the Vitri staining box) and dispense 5 μl of probe onto each slip. Invert slide onto coverslip so that the section contacts the probe solution and picks up the coverslip. The circle scratched on the back of the slide around the section (II,A,5) acts as a useful guide at this stage. The droplet of probe should spread over the entire coverslip with no bubbles. If the section is thick, you may have to add a few extra microliters of probe at one edge of the coverslip with a Gilson-type pipette. Lay slides in hybridization box that contains about 50 ml of hybridization buffer beneath the support rack. If the lid seals well, a vapor of buffer will prevent the probe under the coverslips from drying out and it will not be necessary to seal the edges of the coverslips with mineral oil, as is sometimes recommended (Angerer and Angerer, 1989). Hybridize at selected temperature overnight. To remove coverslips, stand slides in beaker of 4× SSC. Gently agitate slides until coverslips fall off. This may take 10–20 min. Never pry off the coverslips, as this dislodges the sections. Transfer slides to rack and wash in 2× SSC. Wash in 1× SSC at hybridization temperature. Wash in <1× SSC at selected temperature if higher stringency is required. Dehydrate in ethanol (technical grade). Dehydrate in ethanol (AR grade).

B. Autoradiography

1. Materials

X-ray film cassette
Kodak XAR X-ray film
Kodak Liquid X-ray Film Developer Type 2
DuPont Cromex MRF 32 single-coated film
distilled water
Kodak NTB-2 emulsion
Large, airtight, light-proof container (instant coffee tins are ideal) with silica gel
Kodak D19 developer
Kodak unifix
0.25% (w/v) toluidine blue
Merckoglass mounting medium (Merck, D-6100 Darmstadt, Germany)
lead weights (5 g fishing sinkers)

2. X-Ray Film Autoradiography—[32P] and [35S] Probes Only

This type of autoradiography is very simple and gives a useful overview of labeling at a macroscopic level (Fig. 1, see Color Plates). Tape slides, section upward, onto a sheet of stiff paper in X-ray film cassette. Tape additional new slides around your probed slides to form a flat surface. In the darkroom under a red safelight (Kodak Wratten No. 2), place a sheet of Kodak XAR X-ray film over the slides. Expose at room temperature without an intensifying screen for 4–12 h [32P] or 24–72 h [35S]. Develop film at 20°C for 2 min, wash, fix, and rinse under running water. Finer resolution can be achieved with DuPont Cromex MRF 32 single-coated film. Expose for four times the suitable exposure determined with XAR film.

3. Liquid Emulsion Microautoradiography

Microautoradiography provides finer resolution and is useful at the tissue or cell level (Fig. 2, see Color Plates). This procedure can be done after the X-ray film autoradiography has been completed. Liquid emulsion can only be handled under the prescribed safelight (Kodak Wratten No. 2). Since long drying periods are necessary, it is convenient to have a darkroom with double doors or a large light-proof box in which to store slides while they dry. The unused emulsion should be stored in 4-ml aliquots in scintillation vials away from extraneous radiation. Prolonged storage of emulsion increases background. Blank slides should always be included to check the status of the emulsion. Good resolution requires a thin emulsion layer.

Add 2 vol of distilled water to aliquot of Kodak NTB 2 emulsion. Stand in 40°C waterbath for 1 h. Warm a pasteur pipette by gently squirting emulsion in and out several times. Hold slide on slight angle and slowly squirt a small stream of emulsion over the section. Stand the slide on a piece of absorbent paper and allow to dry for 1 h. I find this method more reliable than dipping for obtaining a thin layer. Repeat for all slides. Transfer slides to tray and place in light-proof, airtight container containing silica gel. Expose for suitable period. Develop in Kodak D19 for 2 min at 15°C. Wash gently in water for 2 min at 15°C. Fix in Kodak Unifix at 15°C for 2 min. Wash at 15°C for 30 min in gently running water. Maintenance of constant temperature minimizes wrinkling and cracking of the emulsion. Air-dry at room temperature.

Exposure periods range from about 1 day up to several weeks, depending on the isotope used and the abundance of target. It is best to have several duplicate slides that can be taken out and developed at intervals to check for adequate exposure.
Stain section under a drop of 0.25% toluidine blue for 1 min. Rinse well with water and air dry. Make a permanent mount by placing about 20 µl of Merckoglass on the section, cover with coverslip and weight down with small piece of lead. Allow to harden overnight. Silver grains can be viewed by standard bright field microscopy, under which they appear as small black spots. The silver particles can also be viewed by dark-field optics\(^1\) in which they appear as brilliant white points (see Fig. 2).

VI. Controls

A number of different control experiments can be used to verify the authenticity of the signal. Some very convincing false positives can occur with in situ hybridization, so good controls are essential. Controls peculiar to RNA probes are outlined in Section IV,C. When localizing RNAs, it is useful to predigest some sections with RNase, which should remove most of the signal (digest with 1 mg/ml pancreatic RNase (Boehringer Mannheim, Germany) in 2 mM MgCl\(_2\), 0.1 M Tris–HCl, pH 7.5, at 37°C for 1 h, then wash extensively with 5 mM EDTA, 0.1 M Tris–HCl, pH 7, before air-drying). Similarly, DNase digestion can be used to eliminate DNA targets. An irrelevant probe (i.e., one for which there is no target) can be used in parallel to detect nonspecific binding. When using indirect nonisotopic systems, it is recommended to process several sections in which one of the detection reagents is omitted in order to demonstrate that no spurious signal is being generated. A positive control is very useful for optimizing a new in situ hybridization experiment. Using a probe for an abundant target such as rRNA, or a highly expressed housekeeping gene, can help in optimizing parameters. Checking retention of nucleic acids in your preparations by acridine orange staining (McFadden et al., 1988b) is also recommended.

VII. New Techniques

Recent innovations have opened new avenues for in situ hybridization. Finer resolution and increased sensitivity now allow us to approach new questions. Techniques for in situ hybridization at the EM level are answering questions about endosymbiosis (McFadden et al., 1994) and virology (Bonfiglioli et al., 1994). The use of fluorochromes as labels offers two novel advantages: high resolution and the ability of label different probes with different colors and use them simultaneously (Titus, 1991). The key application of fluorochromes has been in mapping genes on metaphase chromosomes. Sometimes known as chromosome painting, or alternatively as FISH (fluorescent in situ hybridization), this technique now allows visualization of the position of a DNA sequence on

\(^1\)Consult reference O’Brien and McCully (1986) for setting up dark-field optics.
its carrier plant chromosome (Heslop-Harrison et al., 1993; Heslop-Harrison and Bennett, 1990). By using three different colors (i.e., fluorescein, rhodamine, and coumarin) it is possible to map three different DNA elements to a single metaphase spread by triple-label in situ hybridization (Heslop-Harrison et al., 1993). Currently it is possible to detect a 10kb target on a chromosome.

The principle source of noise in in situ hybridization experiments is nonspecific binding of probe to the tissue. The labeled probe provides a signal even though it has not hybridized with a target. One approach to avoid such noise is PRINS (primed in situ labeling). The principal behind this technique is to incorporate the label into a newly formed DNA strand in situ (Koch et al., 1991). The first step involves annealing a primer to the target sequence. The primer is then extended by a polymerase, and the labeled nucleotides are incorporated into the new strand (Koch et al., 1991). In theory, label is only incorporated at sites where the primer has successfully annealed to the target, thereby providing particularly high signal-to-noise ratios from a very rapid procedure (Koch, 1992).

The PRINS technique represents the first footsteps in the development of the latest innovation in in situ hybridization technology—the in situ PCR. The power of the PCR is its ability to turn vanishingly small quantities of DNA into visible amounts. To be able to do an amplification in situ will be an invaluable aid in detecting particularly rare target sequences. A number of papers describing in situ PCRs have now appeared (Nuovo, 1992) and the protocols are relatively simple. Slide griddles—on which the denaturation, annealment, and extension cycles can be performed on the slide—are now available for several brands of thermal cyclers. Like the in vitro PCR, in situ PCRs make use of a thermostable polymerase so that repeated cycles can be undertaken. Two approaches to visualizing the amplification product have been tried. The first involves an initial amplification protocol, then a standard in situ hybridization protocol with a labeled probe designed to detect the PCR products. The second approach is to incorporate a tagged nucleotide into the new strands during the PCR. The tags are then detected with antibodies and visible markers. Increased sensitivity allows detection of single-copy sequences, but like in vitro PCRs the in situ PCR can be tricky to optimize and good controls are essential (Nuovo, 1992).

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References


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Chapter 9, Fig. 3  Immersion immunofluorescence. An oilseed rape leaf protoplast has been labeled with MAC 207 (a), and some suspension-cultured carrot cells have been labeled with JIM8 (b). The immunofluorescence is green. The AGP epitope recognized by MAC 207 is present at the outer surface of the plasma membrane; without fixation, the MAb has caused patching of the plasma membrane antigen. The epitope recognized by JIM8 is present in the cell walls of some of the cells ×440.

Chapter 12, Fig. 1  Macroscopic localization (Section V, B, 2) of mRNA for a type II proteinase inhibitor in *Nicotiana alata* (A) X-ray film autoradiograph (B) Cryosection of pistil stained with toluidine blue. The mRNA is most abundant in the stigmatic tissue (s), the transmitting tract (tt) and cortex (c) are not labeled. A faint signal is present in the vascular bundles (v). The probe was a cDNA labeled with $^{32}$P by random priming. Reproduced from Atkinson *et al.* (1993), with permission.
Chapter 12, Fig. 2 Markedly different patterns of gene expression in the apical meristem of tomato detected by microautoradiography (Section V, B,3). The left panels show bright-field micrographs of wax sections each hybridized with a different $^35$S-labeled RNA probe (A, rpl2, a ribosomal protein; C, Lip1, lipid transfer protein; E, ADC, arginine decarboxylase; G, rpl38, a ribosomal protein; I, H2A, a histone). On the right are the corresponding dark-field images in which the silver grains in the emulsion are visible as brilliant white dots. Reproduced from Fleming et al. (1993), with permission.