

[ *Plant BioTechniques Series (3)* ]  
***In situ* Hybridization to Cellular RNA Using  
<sup>35</sup>S-labeled cRNA Probes**

Taku DEMURA\*\*† and Hiroo FUKUDA\*\*

*In situ* hybridization is a powerful technique to analyse spatial patterns of RNA accumulation at the cellular level. This technique was established and developed in the animal systems. So far, this technique has also been applied to many plant materials and much information on plant gene expression has been obtained. However, it is true that it is not easy to succeed with this technique since a number of factors should be optimized for each material of interest. This technique involves the following steps: (1) preparation of sections, (2) preparation of probes, (3) hybridization, and (4) detection, within each several factors are included. In this issue we describe a procedure using <sup>35</sup>S-labeled cRNA probes, with which we have succeeded in detecting the expression of the vascular tissue-specific genes<sup>1)</sup>.

### 1. Preparation of sections

#### (1) Fixation

Fixation of plant material is an important step of *in situ* hybridization. Underfixation causes poor preservation of tissue morphology and low retention of RNA molecules *in situ*, whereas overfixation limits the accessibility of labeled probes to the target RNA molecules. Therefore, fixative and fixation time should be determined empirically for each material.

(a) Divide the tissues into small pieces in GA/PFA fixative (1% glutaraldehyde, 3% paraformaldehyde, 50 mM sodium phosphate buffer, pH 7.5, 50 mM NaCl)<sup>4)</sup>.

(b) Incubate the tissues in the fixative for 4 h at 4°C with occasional swirling. Degas 2-3 times of 10 min. each during the fixation<sup>5)</sup>.

#### (2) Dehydration and embedding

Dehydration and embedding should be done gradually since drastic changes in concentration cause distortion of tissue morphology.

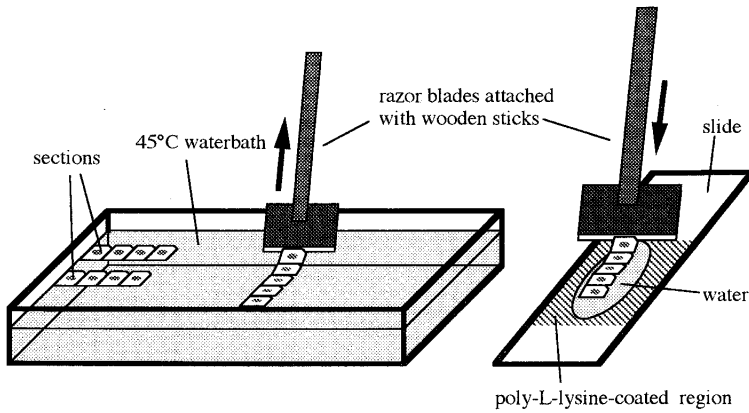
(a) Dehydrate the tissues by incubating in the following ethanol solutions (in water) for 20 min. each at 4°C in succession: 30%, 50%, 70%, 90%<sup>6)</sup>. Rinse the tissues with 100% ethanol and incubate in fresh 100% ethanol for overnight at 4°C. Repeat incubation in fresh 100% ethanol for 20 min. at room temperature. All 100% ethanol should be dehydrated with Molecular Sieves (*e. g.* Wako #134-06095; Nakarai # 233-56).

(b) Incubate the tissues in the following tertiary butyl alcohol (TBA) solutions (in ethanol) for 20 min. each at room temperature in succession: 30%, 60%, 90%<sup>7)</sup>. Rinse the tissues with 100% TBA and incubate twice in fresh 100% TBA for 20 min. at room temperature. Replace the 100% TBA with liquid paraffin (*e. g.* Wako #128-04375) that had been diluted 1: 1 with TBA and incubate overnight at 58°C to evaporate TBA<sup>8)</sup>. Remove the liquid paraffin using a Pasteur pipet and add

\* Biological Institute, Graduate School of Science, Tohoku University, Aramaki, Aoba-ku, Sendai 980-77, Japan

\*\* Botanical Gardens, Faculty of Science, University of Tokyo, Hakusan, Bunkyo-ku, Tokyo 112, Japan

† To whom correspondence should be addressed.



**Fig. 1** Preparation of sections.

Sections floated on a 45°C waterbath can be mounted onto a poly-L-lysine-coated slide using a razor blade attached with a wooden stick.

melted paraffin wax (*e. g.* Paraplast, Oxford) that had been incubated at 58°C. Incubate the tissues at 58°C for 12 h. Repeat this three more times using a hot Pasteur pipet.

(c) Transfer the tissues using hot forceps into embedding molds that had been filled up with the melted paraffin wax<sup>9</sup>. Immediately after the transfer, orient the tissues using hot forceps or a hot needle. Leave paraffin blocks at room temperature to harden completely. Store the paraffin blocks in a dry place at 4°C. The blocks are stable for more than an year.

### (3) Sectioning

Sectioning requires experience and it is recommendable to get advice from experienced persons.

(a) Prepare poly-L-lysine-coated slides by spreading a few drops of 1 mg/ml poly-L-lysine (*e. g.* Sigma #P-1399) in 10 mM Tris-HCl (pH 8.0) using the tip of a pipet. Dry the slides and store at 4°C. The slides can be use for several weeks.

(b) Cut 4- to 10- $\mu$ m sections from the paraffin blocks. Float the sections on a 45°C waterbath to spread (**Fig. 1**). Put a drop of water onto the poly-L-lysine-coated slides on a 40°C heating plate and mount the sections on the slides (**Fig. 1**). Remove the remaining water and incubate the slides overnight at 40°C to attach the sections to the slides. Store the sections in a dry place at 4°C. These sections are stable for up to several weeks.

## 2. Preparation of <sup>35</sup>S-labeled cRNA probe (Fig. 2)

### (1) Template DNA

(a) Insert cDNA of interest into a plasmid containing a T3, T7, or SP6 promoter (*e. g.* pBluescript, Stratagene; pGEM, Promega)<sup>10</sup>.

(b) Linearize 10  $\mu$ g of the plasmid DNA with an appropriate restriction enzyme that produces a blunt or a 5'-protruding end<sup>11</sup>.

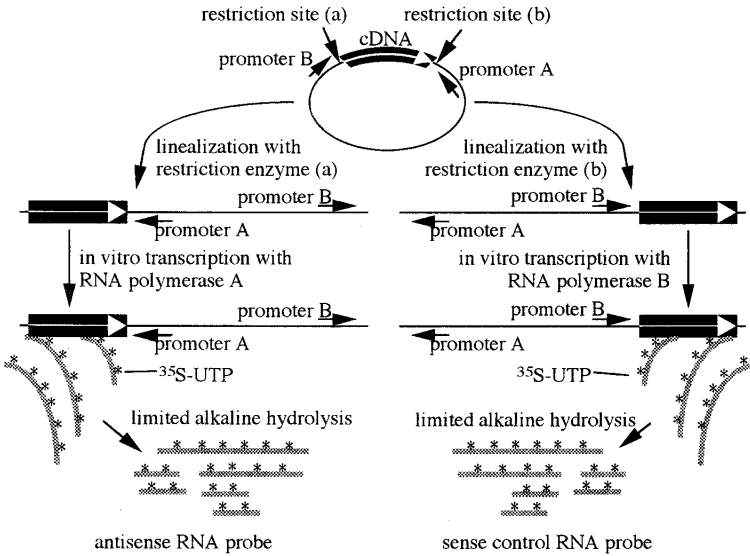
(c) Add RNase-free proteinase K (*e. g.* Gibco-BRL #22530-049) to a final concentration of 0.5  $\mu$ g/ $\mu$ l reaction mixture. Incubate at 37°C for 30 min.

(d) Purify the DNA by two phenol/chloroform extractions and ethanol precipitation.

(e) Dissolve the template DNA at 1  $\mu$ g/ $\mu$ l in TE buffer (10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA).

### (2) *In vitro* transcription

(a) Mix the following at room temperature<sup>12</sup>: 5  $\mu$ l of 5 $\times$  transcription buffer (supplied with RNA polymerase), 1  $\mu$ g of template DNA, 1  $\mu$ l of 10 mM rATP, 1  $\mu$ l of 10 mM rCTP, 1  $\mu$ l of 10 mM rGTP, 1  $\mu$ l of 750 mM DTT, 20-40 U of RNase inhibitor (*e. g.* Takara), 50-200  $\mu$ Ci of [<sup>35</sup>S]rUTP $\alpha$



**Fig. 2** Preparation of  $^{35}\text{S}$ -labeled cRNA probes.

Antisense and sense control probes can be synthesized from a single plasmid clone using appropriate restriction enzymes and RNA polymerases.

S (*e.g.* Amersham #SJ1303 or #SJ603), 10 U of T3, T7, or SP6 RNA polymerase (*e.g.* Stratagene; Promega; NEB), and water to a final volume of  $25\ \mu\text{l}^{13}$ .

(b) Incubate for 30 min. at  $37^\circ\text{C}$ .

(c) Add 10 U additional RNA polymerase and incubate for additional 30 min.

(d) Remove a  $1\ \mu\text{l}$  sample for calculation of percentage incorporation<sup>14</sup>.

(e) Digest the DNA template by adding 10 U of RNase-free DNase I (*e.g.* Stratagene #600031) with  $10\ \mu\text{g}$  of yeast tRNA as carrier and incubating for 15 min. at  $37^\circ\text{C}$ .

(f) Purify the cRNA probe by two phenol/chloroform extractions and ethanol precipitation.

(g) Dissolve the probe in  $50\ \mu\text{l}$  of 15 mM DTT.

### (3) Limited alkaline hydrolysis of probes

Shorter probes appear to penetrate the sections more efficiently than longer, resulting in higher signals<sup>2</sup>. Therefore, the synthesized cRNA probes should be partially hydrolysed to 100–200 bases.

(a) Add  $30\ \mu\text{l}$  of 0.2 M  $\text{Na}_2\text{CO}_3$  and  $20\ \mu\text{l}$  of 0.2 M  $\text{NaHCO}_3$  to the probe ( $50\ \mu\text{l}$ ).

(b) Incubate at  $60^\circ\text{C}$  for the calculated time (in minutes) as following<sup>15</sup>:

$$t(\text{min.}) = (L_0 - L_f) / (KL_0L_f)$$

$L_0$  = starting length (kb)

$L_f$  = final length (kb) = 0.1 to 0.2 (kb)

$K = 0.11$

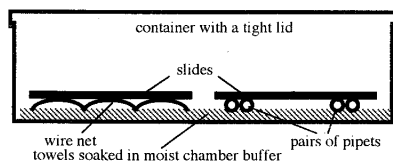
(c) Add  $3\ \mu\text{l}$  of 3 M sodium acetate (pH 6.0) and  $5\ \mu\text{l}$  of 10% glacial acetic acid to stop the reaction.

(d) Ethanol precipitate the probe and dissolve in  $20\ \mu\text{l}$  of 15 mM DTT. Remove a small aliquot of the probe for calculation of quantity and for check of the size on a agarose gel. The probe stored at  $-80^\circ\text{C}$  can be used within 2 weeks.

## 3. Hybridization

### (1) Pretreatment of slides for hybridization

Aims of this step are to increase the accessibility of the labeled probes to the target RNA and to reduce non-specific binding of the probes to the sections and the slides.



**Fig. 3** Moist chamber.

A conveniently sized container with a tight lid (*e. g.* Tupperware), on the bottom of which paper towels (*e. g.* Whatman 3 MM paper) soaked in moist chamber buffer (50% formamide, 300 mM NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA) and a wire net (or pairs of plastic pipets) are placed.

(a) Dewax by incubating slides in xylene for 10 min. Repeat once.

(b) Hydrate through the following solutions for 30 sec. each: 100% (twice), 95%, 85%, 70%, 50%, and 30% ethanol and water (twice).

(c) Immerse the slides into proteinase buffer (100 mM Tris-HCl, pH 7.5, 50 mM EDTA) prewarmed at 37°C. Replace the buffer with fresh proteinase buffer at 37°C. Add RNase-free proteinase K (*e. g.* Gibco-BRL #22530-049) to a final concentration of 1  $\mu\text{g}/\text{ml}$ . Incubate at 37°C for desired time to digest proteins partially<sup>16</sup>. Rinse twice with water to remove proteinase K.

(d) Immerse the slide in freshly prepared 0.1 M triethanolamine (pH 8.0) and incubate for 5 min. at room temperature. Add acetic anhydride at a final concentration of 0.25% (v/v) and mix immediately. Incubate for 10 min. at room temperature<sup>17</sup>. Rinse with 2 $\times$ SSC (1 $\times$ SSC: 150 mM NaCl, 15 mM sodium citrate).

(e) Dehydrate through the following ethanol solutions (in water) for 5 min. each: 30%, 50%, 75%, 95%, 100% (twice). Dry under vacuum for 1 h.

## (2) Prehybridization and hybridization

(a) Prepare prehybridization buffer containing 50% formamide, 300 mM NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 $\times$ Denhardt's solution (0.02% Ficoll, 0.02% PVP, and 0.02% BSA), 100 mM DTT, and 250  $\mu\text{g}/\text{ml}$  denatured herring sperm DNA (or yeast tRNA).

(b) Place the slides in a moist chamber (**Fig. 3**). Apply 100-200  $\mu\text{l}$  of the prehybridization buffer to each slide and incubate at 50°C for 1-2 h. Remove the prehybridization buffer by soaking up the buffer with Whatman 3 MM paper.

(c) During the prehybridization step, prepare hybridization buffer containing 50% formamide, 300 mM NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 $\times$ Denhardt's solution, 100 mM DTT, 250  $\mu\text{g}/\text{ml}$  denatured herring sperm DNA (or yeast tRNA), 10% dextran sulfate, 80-160 U/ml RNase inhibitor, and 1-4 $\times$ 10<sup>8</sup> cpm/ml (100-400 ng/ml) <sup>35</sup>S-labeled cRNA probe<sup>18</sup>. Incubate the hybridization buffer at 80°C for 5 min.

(d) Apply 50-100  $\mu\text{l}$  of the hybridization buffer to each slide, and carefully cover the sections with a coverslip or a small piece of Parafilm. Incubate at 50°C for overnight in the moist chamber.

## (3) Washing

(a) Remove the slides from the moist chamber and soak off the coverslip or the Parafilm by dipping the slides in 4 $\times$ SSC at 50°C. It takes about 10 min. to soak off.

(b) Wash the slides in fresh 4 $\times$ SSC for 10 min. at 50°C with gentle shaking. Repeat this three more times.

(c) Incubate the slides in STE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 500 mM NaCl) for 10 min. at 37°C. Incubate in fresh STE buffer containing RNase A at 20  $\mu\text{g}/\text{ml}$  for 30 min. at 37°C. Wash twice in fresh STE buffer for 10 min. each at 37°C with gentle shaking to remove RNase A.

(d) Wash the slides twice in  $2\times$ SSC for 30 min. each at 50–57°C with gentle shaking. Wash twice in  $0.1\times$ SSC for 30 min. each at 50–57°C with gentle shaking.

(e) Dehydrate through the following ethanol solutions(in water)for 5 min. each: 30%, 50%, 75%, 95%, 100% (twice). Dry under vacuum for 1 h.

#### **4. Detection**

##### **(1) Emulsion autoradiography**

All steps should be carried out in complete darkness or at a reasonable distance from a safety light.

(a) Take 10 g of autoradiographic emulsion(*e. g.* NTB-2, Kodak) using a balance and heat in a 45°C waterbath to melt. After the emulsion has melted, dilute the emulsion with 10 ml of water prewarmed at 45°C and mix gently. Transfer the diluted emulsion into a dipping chamber<sup>19</sup>.

(b) Dip the slides once into the dipping chamber as smoothly as possible(2 to 3 sec. for each dip).

(c) Holding the slide vertically, remove excess emulsion by blotting the bottom edge on a paper towel for a few seconds. Dry the emulsion on the slides by placing the slides vertically in a test tube rack for 1–2 h at room temperature.

(d) Transfer the slides to a light-tight slide box with desiccant and expose at 4°C for desired time<sup>20</sup>.

(e) Warm the slide box containing the slides to room temperature. Develop the slides by dipping in freshly prepared developer(*e. g.* Rendol, Fuji; D-19, Kodak)for 2–3 min. Rinse in 3% acetic acid for 30 sec. Fix in freshly prepared fixer(*e. g.* Renfix, Fuji; Kodak Fixer, Kodak)for 5 min. Rinse in running tap water for about 15 min.

##### **(2) Counterstaining and observation**

(a) Scrap off emulsion on the back of the slide with a razor blade.

(b) Counterstain the sections by immersing the slides in 0.1% toluidine blue for desired time (1–2 min.). Rinse in running tap water until blue color in the emulsion disappears.

(c) Dehydrate through the following ethanol solutions(in water)for 1 min. each: 30%, 50%, 75%, 95%, 100% (twice). Dip twice in xylene. While the slides are still wet, apply a few drops of mounting medium(*e. g.* Enteran New, Merck; Permount, Fisher)and cover the sections with a coverslip. Remove excess mounting medium around the edges of coverslips with 3 MM paper and dry the slides.

(d) Observe the sections using bright-field or dark-field microscopy. The silver grains of hybridization signal will appear black in bright-field or white in dark-field.

#### **5. Troubleshooting**

##### **(1) Poor morphological preservation of sections**

Optimization of fixation conditions might be inadequate. We make a point of preparing a series of fixation time for new samples at first. If changes in the fixation time do not improve the preservation, it is advisable to change the fixative to the others since some fixatives with higher osmolarity might cause plasmolysis resulting in poor morphology.

##### **(2) Small length and poor quantity of cRNA probe**

Preparation of the template DNA and/or *in vitro* transcription might be problems. While DNA purified on CsCl gradients is recommended, we routinely use DNA that has been prepared by alkaline lysate procedures. In this case, contaminating RNA molecules should be removed by RNase A that, in turn, should not be contaminated in the template DNA. Be sure to treat with Proteinase K before purification of the template DNA. Next, check that the correct RNA

polymerase is used and that the activity of the polymerase is intact.

### (3) Low signal and high background

Overfixation prevents the labeled probes from penetrating the sections efficiently; shorten the fixation time. Non-specific binding of probes can sometimes be lowered by using a fresh batch of [<sup>35</sup>S]rUTP stored properly at -80°C, by increasing the temperature of the washing up to 65°C, and by selecting a different portion of the cDNA as probe.

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## References and Notes

- 1) Demura, T., H. Fukuda, 1994. *Plant Cell*, **6**: 967-981.
- 2) Cox, K. H., *et al.*, 1984. *Dev. Biol.*, **101**: 485-502.
- 3) Cox, K. H., *et al.*, 1986. *J. Mol. Biol.*, **188**: 159-172.
- 4) Other fixatives containing formaldehyde, glutaraldehyde (GA), or paraformaldehyde (PFA) at a concentration of 1-4% and buffered at pH 7.0-8.0 with sodium phosphate, sodium cacodylate trihydrate, or PIPES are available.
  - a) 0.25% GA, 4% PFA, and 50 mM PIPES (pH 7.5) at 4°C for 4 h (for carrot somatic embryos and seedlings).
  - b) 1% GA, 50 mM sodium cacodylate trihydrate (pH 7.0) at room temperature for 3 h (for tobacco leaves, stems, roots, petals, ovaries and anthers, maize leaves and soybean roots and embryos).
  - c) 3.7% formaldehyde, 50% ethanol, 5% acetic acid (FAA fixative) at room temperature for 3-5 h (for *Arabidopsis* tissues).
  - d) 1.85% formaldehyde, 45% ethanol, 5% acetic acid (modified FAA fixative) at 4°C for overnight (for rice tissues).
- 5) The degassing should be done by bringing the vacuum up and down slowly, or else tissue morphology will be destroyed.
- 6) When the FAA fixatives are used, the dehydration step starts with 50% ethanol.
- 7) Xylene can be used in place of TBA. However, since xylene is a toxic organic solvent, the treatments with xylene should be carried out in a hood.
- 8) Alternatively, add paraffin wax to approximately half of the volume of TBA (xylene), leave overnight at room temperature, and incubate at 42°C until the paraffin wax is melted completely.
- 9) Embedding molds made of silicone (*e. g.* DSK; Ladd; TAAB), stainless, and porcelain are useful. Small (35 mm) plastic dishes for tissue culture (*e. g.* Nunc; Sumitomo Bakelite) are also available.
- 10) It is recommended to insert the cDNA into the multi-cloning site of the plasmid from which antisense and sense (control) probes can be synthesized using the appropriate RNA polymerase (*e. g.* pBluescript; pGEM).
- 11) Restriction enzymes that produce 3'-protruding ends should be avoided since non-specific transcription for synthesis of opposite strand can occur from the ends.
- 12) Avoid incubating on ice since the DNA can sometimes precipitate by the spermidine contained in the transcription buffer.
- 13) Adding 200  $\mu$ Ci of 1000 Ci/mmol [<sup>35</sup>S]rUTP, which is equivalent to 200 pmol, to a 25  $\mu$ l reaction mixture produces a rUTP concentration of 8  $\mu$ M.
- 14) Under this condition 50-90% of the label will be incorporated resulting in about 150-250 ng (200 pmol  $\times$  50-90%  $\times$  4  $\times$  345 g) of labeled cRNA with specific activities of over 10<sup>6</sup> cpm/ng.
- 15) For instance; starting length = 1.2 (kb), final length = 0.2 (kb),  $t(\text{min.}) = (1.2 - 0.2) / (0.11 \times 1.2 \times 0.2) = 1.0 / 0.0264 = \text{about } 38 (\text{min.})$ .
- 16) Incubation time ranging from 15 to 45 min. might be adequate for most of the plant materials to digest proteins partially without destroying tissue morphology or causing the sections to fall off the slides during

the following steps.

- 17) Acetylation of the sections in this treatment reduces non-specific binding of the probes.
- 18) Cox *et al.* (1986) described that the final probe concentration at about 300 ng/ml per kb of probe was required for saturation that causes the maximum hybridization efficiency.
- 19) Do not use old emulsion (more than 2 months after purchase), which may cause an unacceptable background. Hypercoat LM-1 (Amersham #RPN40) is also available without dilution.
- 20) The time of exposure varies with the mRNA abundance in the tissue and the probes. Prepare a series of test slides and develop it at first to monitor the efficiency of exposure.