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In Situ Hybridization on Paraffin Sections

This protocol is derived from Jackson (1991) and Lincoln et al. (1994) with slight modifications.

1. Probe preparation by *in vitro* transcription

1.1. Preparation of template plasmid

1. Linearize template plasmid using an appropriate restriction enzyme. Ensure complete digestion. Choose an enzyme that creates either 5' overhang or blunt end, but not 3' overhang.
2. Extract DNA twice with phenol/chloroform and once with chloroform. Precipitate DNA with ethanol and sodium acetate. Rinse pellet with 70% ethanol. Dissolve in 20ul RNase-free water.

1. 2. *In vitro* transcription

1. Prepare reaction as follows. Incubate reaction at 37°C for 2 hrs.

linearized DNA	2 ug
*DIG RNA labeling mix	2 ul
*10 x transcription buffer	2 ul
RNase free water	
*Protector RNase inhibitor (40 U/ul)	1 ul
*RNA polymerase (20-40 U/ul)	2 ul (mix gently)
Total	20 ul

*Supplied from Roche Applied Science.

2. Run 1ul of the reaction on a 2% agarose gel for 15min to check the product.
3. Add 1ul DNase I. Incubate at 37°C for 10 min.
4. Stop reaction by adding following reagents:

0.5M EDTA	1 ul
8M LiCl	1 ul
tRNA (10mg/ml)	1 ul
ethanol	60 ul

5. Incubate at -20°C for 30 min and centrifuge at maximum speed for 30 min. Rinse pellet with 70% ethanol. Briefly dry pellet (do not dry completely). If you omit alkaline hydrolysis (**1.3**), dissolve pellet in 50 ul water. Use 1 ul for estimation probe concentration (**1.4)** and store the rest at -20°C.

1. 3. Alkaline hydrolysis (optional)

In this step, probe is hydrolyzed to obtain smaller fragments of about 75 to 150 nt in length. This improves probe penetration into tissues. However, we routinely obtain good results without this treatment for probes about 500 to 1,000 nt long when working with *Arabidopsis* tissues.

1. Dissolve pellet and prepare following reaction:

RNase free water	45 ul
0.1M DTT	5 ul
2 x hydrolysis buffer	50 ul

   (80 mM $NaHCO_3$, 120 mM Na_2CO_3, freshly prepared)
2. Incubate at 60°C for calculated time according to the formula as follows:

   $t = (Li – Lf) / (k \cdot Li \cdot Lf)$

   where t = time (min), k = 0.11 (nt / min), Li = initial probe length (nt), Lf = final probe length (nt)
3. Add 10 ul of 3M Na acetate, 5 ul of tRNA (10 mg/ml) and 300 ul of ethanol. Incubate at -20°C for 30 min and centrifuge at maximum speed for 30 min. Rinse pellet with 70% ethanol. Briefly dry pellet (do not dry completely). Dissolve in 50 ul water and use 3 ul for checking probe length with electrophoresis. Use 1 ul for estimation of probe concentration (**1.4)** and store the rest at -20°C.

1. 4. Estimation of probe concentration

Carry out the following steps at room temperature.

1. Using 1 ul of probe, prepare dilution series of 1/100, 1/200, ···1/6400 with 50 ug/ml tRNA as diluent. As a control, use DIG-labeled Control RNA (Roche). Spot 1 ul each on nylon membrane.
2. Soak the membrane in TBS (see **Reagents**) briefly.
3. Incubate for 30 min in buffer 1 (see **Reagents**).
4. Incubate for 30 min in TBS + Anti-DIG-AP (Roche 1093274; 1:5000 dilution).
5. Wash twice in TBS for 15 min each.
6. Incubate for 5 min in buffer 3 (see **Reagents**).
7. Incubate in Western Blue (Promega; use the amount just enough to cover the membrane) in dark. Check filter every 5 min until optimal staining. Stop staining by washing filter with water.
8. Determine the probe concentration by comparing the spots with those of the control.

2. Tissue preparation and sectioning

2. 1. Fixation

Day 1. Tissues are fixed in 4% paraformaldehyde (PFA) + 4% DMSO in PBS (see **Reagents)** at 4°C. For some tissues (e.g. *Arabidopsis* inflorescence), fixation with FAA (10% formalin, 50% ethanol, 5% acetic acid) at room temperature for 4 hrs equally works. To prepare PFA fixative, add 50 ul of 1N NaOH and 2g of PFA to 43 ml of water in 50 ml plastic tube. Incubate at 70°C until PFA dissolves. Cool down the tube on ice and add 5ml of 10x PBS and 2ml of DMSO. To make FAA, simply mix formalin, ethanol, acetic acid and water at the concentration described above. Always use freshly made fixative.

1. Cut tissue into small pieces and immediately put them into fixative placed on ice. At least 20 times volume of fixative relative to tissue volume is needed.
2. Apply vacuum to samples until fixative starts to make small bubbles. Just before fixative starts to boil, release the vacuum rapidly, take the sample out of the chamber and swirl it. Repeat this procedure until tissue sink.
3. Replace fixative and keep samples overnight at 4°C.

2. 2. Dehydration

Day 2. All steps are carried out at 4°C with gentle shaking.

1. Wash with PBS for 30 min x2. If FAA is used as a fixative, wash with 50% ethanol twice for 30 min at 4°C and then start dehydration from 60% ethanol.
2. Dehydrate through ethanol series (30/40/50/60/70/85/95%) for 60 min in each step. If necessary, samples are stored in 70% ethanol at 4°C for several month.
3. Keep samples in 95% ethanol overnight.

2. 3. Dehydration (continued)

Day 3. All steps are carried out at room temperature with gentle shaking. The ethanol used from the following steps on is prepared anhydrous by using molecular sieve 3A. Complete removal of water is particularly important for obtaining good paraffin sections. For details, see <http://www.bio.umass.edu/microscopy/mol_sieves.htm>.

1. 100% ethanol for 30 min x2.
2. 100% ethanol for 60 min x2.
3. 25% lemosol, 75% ethanol for 30 min.
4. 50% lemosol, 50% ethanol for 30 min.
5. 75% lemosol, 25% ethanol for 30 min.
6. 100% lemosol for 60 min x 2.
7. 100% lemosol and 1/4 volume of paraffin chips (Paraplast Plus; McCormick Scientific) overnight.

2. 4. Paraffin infiltration and embedding

1. Day 4. Put samples at 60°C until paraffin completely melt. Add 1/4 volume of

paraffin chips and leave for a few hours until paraffin completely melt.

2. Replace lemosol/paraffin mix with freshly melted paraffin and leave overnight.
3. Day 5 to 7. Change paraffin twice a day with at least a few hour interval.
4. Day 8. Put an aluminum mold on a hot plate heated to 60°C. Pour paraffin and tissue into it. When necessary, arrange the tissue appropriately by using a dissecting needle preheated with an alcohol lamp. Carefully move the mold to a cool surface to solidify the tissue within the paraffin. Embedded tissues in paraffin blocks can be stored for several years at 4°C

2. 5. Sectioning

Cut sections of 8 um thick and attach them onto precoated glass slides. Write sample name on a frosted part of the slide using a clean pencil. For details and tips for sectioning, see Ruzin S. E (1999).

3. Hybridization and probe detection

3.1. Section pretreatment

1. Place slides onto a slide holder. In each of the following treatments, move the holder up and down several times. Each treatment is carried out at room temperature unless otherwise noted.
2. De-wax with lemosol for 10 min x 2.
3. 100% ethanol for 1 to 2 min x 2. Move the holder up and down until the trace of lemosol cannot be seen.
4. Dehydrate through ethanol series (95/90/80/60/30%) for 1 to 2 min in each step.
5. Water for 1 to 2 min.
6. 2x SSC for 15 min
7. Rinse with water.
8. Proteinase K (1 ug/ml) in PK buffer (see **Reagents**) at 37°C for 30 min. The buffer should be prewarmed. Add proteinase K stock solution into the buffer just before the treatment.
9. Glycine (2 mg/ml) in PBS (freshly prepared) for 2 min.
10. PBS for 2 min x 2.
11. 4% PFA in PBS (freshly prepared) for 10 min.
12. PBS for 5 min x2.
13. Dehydrate through ethanol series (30/60/80/90/95/100%) for 1 to 2 min in each step.
14. Air dry the slides on paper towels.

3.2. Hybridization

1. Observe sections under a binocular and allocate probes to each slide. Write the name

of the probe onto each slide using a clean pencil.

2. Prepare a humidity chamber. Place paper towels wet with 50% formamide, 0.3M NaCl on the bottom. Put plastic spacers onto the towels. Slides will be put on these spacers (**3.2.9**), so that they do not directly touch the wet towels. Prewarm the chamber to 45°C.
3. Prepare hybridization solution as follows (for 15 slides):

RNase free water	400 ul
50% dextran sulfate (w/v)	1000 ul
10x salts	500 ul
deionized formamide	2000 ul
50x denhardt's solution	100 ul
total	4000 ul

4. Dispense 160 ul aliquots to 1.5 ml tubes. Prewarm to 45°C.
5. Prepare probe solution for each slide as follows:

probe	calculated amount*
RNase free water	
50 mg/ml tRNA	4 ul
deionized formamide	20 ul
total	40 ul

*The standard amount of probe is 0.1 ng per 1ul of hybridization solution per 1kb of probe. If a probe is 0.5 kb long, it is 0.1 x 200 x 0.5 = 10 ng (note that the amount of hybridization solution is 200 ul). For a new probe, test 5 times higher or lower.

6. Heat probe to 80°C for 2 min, cool down on ice for 2 min, spin down and keep them on ice.
7. Add probe to hybridization solution and mix thoroughly (either with a vortex mixer or a pipette). Avoid making bubbles.
8. Put slides on a hot plate preheated to 45°C. Apply probe on each slide and cover it with a piece of parafilm (cut to a size just fit to the clear area of the slide). Avoid making bubbles.
9. Put each slide on the spacers in the humidity chamber, tightly close the lid and place the chamber at 45°C overnight.

3. 3. Probe wash

In the following steps, occasionally move the slide lack up and down.

1. Prewarm 0.2x SSC to 55°C and NTE (see **Reagents**) to 37°C.
2. Dip slides into 0.2x SSC. Wait until the parafilm sheet naturally detaches. Move slides to a slide rack in another bottle with 0.2x SSC.

3. Wash in 0.2x SSC for 60 min x 2 at 55°C.
4. NTE at 37°C for 5 min x2
5. RNase treatment (20 ug/mg RNase A in NTE) at 37°C for 30 min.
6. Wash in NTE at 37°C for 5 min x2.
7. Wash in 0.2x SSC for 60 min at 55°C.
8. PBS at room temperature for 5min.

3. 4. Probe detection

1. Put buffer 1 (see **Reagents**) in a plastic container and place the slides. Use the buffer just enough to cover the slides. We routinely use square plastic plates (14 x 10 cm), which can load five slides each. Incubate the slides at room temperature for 45 min, with gentle agitation using an orbital shaker.
2. Replace buffer 1 with buffer 2 (see **Reagents**) and incubate the slides at room temperature for 45 min with gentle agitation using an orbital shaker.
3. Take each slide out, drain excess of buffer 2 and put it on paper towels. Apply Anti-DIG-AP in buffer 2 (1:1000 dilution) and cover it with a piece of parafilm. Put slides in a humidity chamber similarly prepared as in **3.2.2** but with water-wet paper towels, and incubate for 2 hrs at room temperature or overnight at 4°C.
4. Put each slide in buffer 2 and allow the parafilm to detach.
5. Wash 4x in buffer 2 for 20 min each at room temperature, with gentle agitation by an orbital shaker.
6. Replace buffer 2 with buffer 3 (see **Reagents**) and incubate the slides for 5 min x 2.
7. Apply Western Blue (Promega) on each slide and put cover slip. Drain excess amount of liquid and place slides in a humidity chamber.
8. Monitor slides and take photograph when staining is optimum. Staining time depends on probe (6 hrs to 3 days, mostly 1 day).

Reagents

PBS (pH 7)

30 mM NaCl, 7 mM Na_2HPO_4, 3m M NaH_2PO_4

10x stock (1000ml)

NaCl	76 g
$Na_2HPO_4 \cdot 12H_2O$	25 g
$NaH_2PO_4 \cdot 2H_2O$	4.7 g

PK buffer

100 mM Tris-HCl (pH8), 50 mM EDTA

10x salts for hybridization solution

3M NaCl, 100 mM Tris-HCl (pH8), 100 mM Na phosphate (pH6.8), 50 mM EDTA

NTE

0.5 M NaCl, 10 mM Tris-HCl, 1 mM EDTA

TBS

100 mM Tris-HCl (pH7.5), 150 mM NaCl

Buffer 1

1% blocking reagent (Roche) in TBS.

Make fresh by diluting 10% blocking reagent stock (see manufacture's instruction) using TBS.

Buffer 2

1% BSA, 0.3% Tween 20 in TBS

Make fresh by adding BSA powder and Tween 20 to TBS.

Buffer 3

100 mM Tris-HCl (pH9.5), 100 mM NaCl

References

1. Jackson, D. (1991). *In situ* hybridization in plants. *In* Molecular Plant Pathology: A Practical Approach, D.J. Bowles, S.J. Gurr, and M. McPherson, eds (Oxford: Oxford University Press), pp. 163-174.
2. Lincoln C., Long J., Yamaguchi J., Serikawa K., Hake S. (1994). A *knotted1*-like homeobox gene in Arabidopsis is expressed in the vegetative meristem and dramatically alters leaf morphology when overexpressed in transgenic plants. Plant Cell **6**: 1859–1876.
3. Ruzin S. E. (1999). Sectioning and mounting. *In* Plant Microtechnique and Microscopy. Oxford: Oxford University Press), pp. 73-85.