Non radioactive *in situ* hybridization

The following is a non-radioactive *in situ* protocol for plants, using an RNA probe. It is derived from several protocols, compiled and worked out by Cindy Lincoln and then slightly modified by myself. The fixation/dehydration/embedding portion is derived from a protocol from Elliot Meyerowitz's lab. The RNA probe synthesis is from David Jackson. The actual *in situ* section is from both David Jackson and Vivian Irish.

An excellent reference for this protocol is:

Jackson, D. (1991). *In-situ* hybridisation in plants. Molecular Plant Pathology: A Practical Approach. Eds. Bowles, D. J., S. J. Gurr and M. McPherson. Oxford University Press.

Good Luck,

Jeff Long

I. Fixation/Dehydration/Embedding

DAY 1:

Fixative: 4% (w/v) paraformaldehyde; 4% (v/v) DMSO; in 1xPBS

Make up required amount of 1xPBS and pH to 11 with NaOH. Heat to 60-70°C. Add paraformaldehyde (in fume hood). Paraformaldehyde should dissolve within a minute or so. Place on ice. When cooled to 4°C, pH to 7 with H_2SO_4. Add DMSO to 4% (v/v).

Collect tissue into ice-cold fixative. Apply vacuum to samples until paraformaldehyde starts to bubble. Hold vacuum for 15 min and release slowly. Repeat until tissue begins to sink. Replace fixative and gently shake overnight (~12 hrs) at 4°C.

NOTE: Some tissue will never sink using this method (e.g. flowers) because of air spaces in the tissue.

DAY 2:

All steps at 4°C and shaking

__ 1X PBS wash 30 min

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__ 30% EtOH 60 min

__ 40% EtOH 60 min

__ 50% EtOH 60 min

__ 60% EtOH 60 min

__ 70% EtOH 60 min (you can stop here and store tissue for several months in 70% EtOH)

__ 85% EtOH 60 min

__ 95% EtOH+eosin (until light pink; to visualize tissue) overnight

DAY 3:

Room temp and shaking

__ 100% EtOH+eosin 30 min

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__ 25% histoclear, 75% EtOH 30 min

__ 50% histoclear, 50% EtOH 30 min

__ 75% histoclear, 25% EtOH 30 min

__ 100% histoclear 60 min

__ 100% histoclear 60 min

__ 100% histoclear + 1/4 volume paraplast chips overnight (no shaking)

DAY 4:

__ place at 42°C until chips melt completely

__ Add 1/4 volume of chips until completely melted

__ Move to 60°C for several hours

__ Replace wax/histoclear with freshly melted wax overnight (60°C)

DAY 5:

__ __ 2 wax changes (separated by several hours)

DAY 6:

__ __ 2 wax changes (separated by several hours)

DAY 7:

__ __ 2 wax changes (separated by several hours)

DAY 8:

Place tissue in molds. Store at 4°C.

II. Sectioning

Sections are 8μm thick. Slides used are ProbeOn Plus from Fisher Biotechnology. They are pre-cleaned and charged. They also have a white frosting on them that allows you to sandwich them together later in the hybridization/detection steps of the protocol.

Pre-warm slide warmer to 42°C. Place slide on RNase-free slide warmer and apply several drops of DEPC-treated water. Float ribbon of tissue ("shiny" side down) on top of water and allow to sit for a few minutes (we use paintbrushes to handle the ribbons). During this time the ribbon should flatten out.

Drain off water using a Kimwipe (excess water will cause bubbles). Allow slides to incubate on slide warmer overnight so that tissue adheres to slide. Sectioned tissue can be stored with desiccant for several weeks at 4°C.

III. *In vitro* transcription

Linearize plasmid to give run off transcripts with the appropriate polymerase. Use an excess of enzyme/time (i.e. 5X/4hours) to ensure cutting is to completion. Do not use enzymes that leave a 3' overhang. Phenol/chloroform extract twice and precipitate to get rid of any RNases.

Resuspend at 0.5μg/μl in DEPC-treated H_2O. Set up transcription reaction as follows:

DNA 2 μg 4μl
5x buffer 5μl
5Xnucleotides 5μl
RNase inhibitor to 1unit/μl
RNApolymerase to 0.4 units/μl
H_20 to total
TOTAL 25μl

Incubate at 37°C 30 to 60 minutes.

Run 1 μl on a minigel at <100V for roughly 15 minutes as RNA degrades quickly (treat gel box and comb with 0.2N NaOH for 30 minutes before use). Add 75 μl H_20, 1 μl 100mg/ml tRNA 5units RNase-free DNase. Incubate for 10 minutes at 37°C. Add equal volume 4M NH_4Ac and 2 volumes EtOH. Precipitate at -20°C.

Spin down RNA pellet (it should be large). Rinse pellet with 70% EtOH. Air dry or speed vac with no heat.

5X nucleotides are 2.5mM each in H_2O. We use a 1:1 dig-UTP:regular UTP. We get our unlabelled nucleotides from Stratagene, and our dig-UTP from Roche.

Carbonate hydrolysis:

You now want to "chop" up your probe into pieces between 75 and 150 bp long. We typically calculate the reaction for 150 bp final length.

The formula is as follows:

$$Time=Li-Lf/(K \bullet Li \bullet Lf)$$

Li=initial length of probe (in kb)

Lf=final length of probe (0.15 kb)

$K=0.11\ kb^{-1}min^{-1}$

Resuspend pellet in 100μl H_2O. Add 100 μl 2XCO3- buffer. Incubate at 60°C for calculated time Neutralize with 10μl 10% acetic acid. Add 1/10 volume 3MNaAc (pH 5.2) and 2 volumes EtOH. Precipitate at -20°C. Rinse pellet with 70% EtOH. Resuspend in 50% formamide at 1μl/slide for convenience.

A crude way to estimate how much probe you have made is to compare the intensities of the RNA band to that of the DNA template band on your gel. If they are equal, you have synthesized roughly 2 μg of RNA probe.

Use probe at a final concentration in the hybe of 0.5ng/μl/kb. For a new probe try up to 5X higher or lower to find the best concentration. Fisher Probe On Plus slides require 100μl probe per slide. So, if probe is 0.5kb, you need 0.5X100X0.5=25ng probe/slide. Thus 2μg of probe is sufficient for 80 slides.

IV. *In situ* section pretreatment

Make all solutions RNase-free (I don't DEPC-treat most of my solutions or water supply, but I do autoclave everything before I use it.) Treat plastic containers with 0.1M NaOH overnight and rinse with sterile water. Bake glassware and stirbars.

1. Deparaffinize/dehydrate sections:

2X 10 min histoclear

2X 1-2 min 100% EtOH

1-2 min 95% EtOH

1-2 min 90% EtOH

1-2 min 80% EtOH

1-2 min 60% EtOH

1-2 min 30% EtOH

1-2 min H_2O

2. 15-20 min 2X SSC room temp

3. 30 min proteinase K (1μg/ml) in 100 mM Tris pH 8, 50 mM EDTA at 37°C

(Prewarm Tris/EDTA solution to 37°C-add proteinase K from 10 mg/ml frozen stock immediately before adding slides.)

4. 2 min 2 mg/ml glycine in PBS room temp

5. 2 min PBS room temp

6. 2 min PBS room temp

7. 10 min 4% (w/v) paraformaldehyde in PBS pH 7 (made fresh) room temp

8. 5 min PBS room temp

9. 5 min PBS room temp

10. 10 min 0.1 M triethanolamine (made fresh to pH 8) and acetic anhydride.

Elevate slide rack in container of triethanolamine with stir bar. Dispense acetic anhydride (4 mls in 800 mls triethanolamine) into triethanolamine a few minutes before putting slides in so that it is mixed well.

11. 5 min PBS room temp

12. 5 min PBS room temp

13. dehydrate:

30 sec 30% EtOH

30 sec 60% EtOH

30 sec 80% EtOH

30 sec 90% EtOH

30 sec 95% EtOH

2X 30 sec 100% EtOH

14. Store slides in container with a small amount of EtOH in the bottom for up to several hours at 4°C.

V. *In situ* hybridization

1. Decide which slides to use with which probes. Determine how much hybe solution to make based on the total number of slide PAIRS.

Hybe Solution (enough for 5 slide PAIRS)

100 μl 10X in situ salts

400 μl deionized formamide

200 μl 50% dextran sulfate

20 μl 50X Denhardts solution

10 μl tRNA (100 mg/ml)

70 μl H_20 (DEPC-treated)

total volume: 800 μl

This solution is very viscous from the dextran sulfate. I recommend either warming it up before using it, or making more than you need due to loss in dispensing.

2. Air dry slides on clean paper towels or kimwipes- must be completely dry.

3. For each pair of slides, probe should be added to 50% formamide such that the volume is 40 μl. Heat to 80°C for 2 min, ice, spin down and keep on ice.

4. Add 160 μl of hybe for each pair of slides such that the volume is now 200 μl (hybe+probe) for each slide pair (ratio of hybe to probe is 4:1). Mix without generating bubbles.

5. Apply probe

This step is a matter of what works for you. 200 μl of the hybe/probe needs to be added to each slide pair. One technique is to apply 100 μl to each slide, spreading it out over the entire slide withthe side of a pipette tip so that all of the tissue is exposed to probe. Then slowly sandwich

these together. Another is to put all of the probe in the middle of one slide and slowly massage the other slide on top until the two slides are together.
These methods only work for the Probe-On Plus slides from Fisher, which can be sandwiched.

6. Elevate slides above wet paper towels in plastic container (which seals tightly) using plastic pipettes. Hybridize between 50-55°C overnight.

VI. *In situ* posthybridization

Warm 0.2X SSC to 55°C (need ~3L).

Warm NTE solution to 37°C (need ~3L).

1. Dip pairs of slides into prewarmed .2X SSC to separate and rinse them before placing in rack.

2. Wash 60 min in .2X SSC with gentle agitation.

3. Repeat .2X SSC wash 60 min.

4. 5 min NTE prewarmed to 37°C with gentle agitation.

5. Repeat NTE wash 5 min.

6. 30 min RNase (20 μg/ml RNase A in NTE) 37°C with gentle agitation.

7. 5 min NTE 37°C with gentle agitation.

8. Repeat NTE wash 5 min.

9. 60 min .2X SSC 55°C with gentle agitation.

10. 5 min PBS room temp (or 4°C overnight if want to stop here).

11. Place slides on bottom of large plastic container.

45 min wash with 1% Boehringer block (made fresh following their directions) in 100 mM Tris pH 7.5, 150 mM NaCl. Use just enough to cover slides and place on rocking platform at room temp.

12. Replace block solution with 1.0% BSA in 100 mM Tris pH 7.5, 150 mM NaCl, 0.3% Triton X-100 for 45 min as in step 11.

13. Dilute anti-dig antibody (1:1250) in the BSA/Tris/NaCl/Triton solution from step 12.

Make a puddle of antibody solution in a plastic weigh dish. Sandwich slides together and allow capillary action to pull up solution. Drain on Kimwipe and repeat, try to avoid bubbles.

14. Elevate slides above wet paper towels in plastic container and allow to sit at room temp for 2 hours.

15. Drain slides on Kimwipes and separate. Place on bottom of plastic container as in step 11. Wash 4X in BSA/Tris/NaCl/Triton solution, 15 min each, room temp, rocker platform.

16. 10 min 100 mM Tris pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$

17. Dip each slide in Tris pH 9.5/NaCl/$MgCl_2$ solution to ensure all of detergent is washed off.

18. Prepare substrate solution immediately before using:

22 μl NBT
16 μl BCIP
in 10 ml Tris pH 9.5/NaCl/$MgCl_2$ solution (this is enough for 25 slides). Sandwich slides and draw up solution as in 13. Repeat.

19. Place slides in plastic container above wet paper towels in total darkness for 1-3 days.

20. Drain, separate slides, rinse in TE to stop reaction.

21. Dehydrate

Keep time in EtOH to a minimum (i.e. 5 sec each solution). Color product is soluble.

30% EtOH

50% EtOH

70% EtOH

85% EtOH

95% EtOH

2X 100% EtOH

2X histoclear

Drain and mount slides in mounting medium. We use Cytoseal 60 from Stephens Scientific.

VII. Modifications

I have made a couple of changes to the protocol which seem to have helped under certain circumstances (i.e. different tissues/probes). Instead of putting them into the protocol (which works for most things) I have listed them below.

Fixation:

I have routinely been stopping the tissue processing at the 70% EtOH step and just storing the tissue like that when I don't need it right away, or want to stockpile it for one big batch to go into

wax. I have kept tissue in 70% EtOH for over 6 months, with no noticeable loss in signal. When I resume the processing, I do not stop at 95% for overnight, I just continue into the 100% and then histoclear. For really large samples (like short day grown plants) I add a couple of days of wax changes (usually 2 days=4 wax changes). This seems to help with the infiltration of the wax into the center of these tissues and does not seem to effect signal.

Sectioning:

If the samples are large, sometimes they tend to buckle as they come off of the microtome, and the large buckles do not always flatten out when put on the water on the slide. To alleviate this, section the tissue while it is still a little cold. The ribbons may not be as nice, but the individual sections come off much better.

Transcription reactions:

If you are not getting much probe from your reaction, you can spike it with more enzyme and allow it to go on for another 30 to 60 minutes. We have found that T7 and SP6 give us much more probe than T3 in general.

Post hybridization:

We routinely skip the RNase step and the NTE washes on the second day. This has had little affect on the amount of background and seems to enhance the signal for weak probes. However, if high background is a problem, or your probe contains significant homology to another gene, I would recommend keeping the RNase step in.

Color detection:

We have switched over to a room temperature stable premix of NBT/BCIP from Promega called Western Blue (cat# S3841) to which I add Levamisole (Sigma #L9756) to 1mM to cut down on background. This has given us much more consistent results than making up the NBT/BCIP ourselves, although it is more expensive.

Another substrate that I have used for the alkaline phosphatase is Fast Red (Sigma #F4523) which deposits a red precipitate. However, it does not seem to be as sensitive as NBT/BCIP and cannot be exposed to EtOH as it is highly soluble.

Recommended Stock Solutions:

10X PBS pH 7.0:
1.3 M NaCl
70 mM Na_2HPO_4
30 mM NaH_2PO_4

5X NTE:
2.5 M NaCl
50 mM Tris pH 8
5 mM EDTA

20X SSC:
3 M NaCl
300 mM Na Citrate

100 mM Tris pH 8/ 50 mM EDTA

100 mM Tris pH 7.5/150 mM NaCl

Tris pH 9.5

10X PBS/Glycine pH 7 -store at 4°C:
1.3 M NaCl
70 mM Na_2HPO_4
30 mM NaH_2PO_4
20 mg/ml Glycine

2XCO3- buffer -store at -20°C:
80 mM $NaHCO_3$
120 mM Na_2CO_3

RNase A -store at 4°C:
20 mg/ml

10X in situ salts -store at room temp:
3 M NaCl
100 mM Tris pH 8
100 mM Na Phosphate pH 6.8
50 mM EDTA

tRNA -store at -20°C:
100 mg/ml

Dextran Sulfate -store at -20°C:
50% (w/v) in DEPC-treated water

Proteinase K -store at -20°C:
10 mg/ml

50X Denhardts -store at -20°C