Non Radioactive *in situ* Hybridization with *Arabidopsis* Floral Tissue

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Modified and updated by Maxi Oelschner.

1 Fixation and Embedding

1.1 Fixation

We obtained the best signals with FAA (Formaldehyde/Acetic Acid) as fixative.
Always make fresh:

	for 10ml (volume for one vial)
Ethanol 100% p.a.	5 ml (50% final)
Glacial Acetic Acid 100%	0.5 ml (5% final)
Formaldehyde 37%	1.0 ml (3.7% final)
Water	3.5 ml

Harvest the flowers and transfer to the fixative (in glass vials or falcon tube).
Transfer plant material into white cassettes and label them with a pencil. Close them with a lid.

Transfer the cassettes into a tray and this one in the Leica ASP200 (automated vacuum tissue processor).
Start program "routine overnight":

station	reagent	duration	temp	drain (seconds)
1	FAA-fixative	4 hours		140
2	ethanol 70% denatured	1 hour		120
3	ethanol 90% denatured	1 hour		120
4	ethanol 90% denatured	1 hour		120
5	ethanol 100% denatured	1 hour		120
6	ethanol 100% denatured	1 hour		120
7	ethanol 100% denatured	1 hour		140
8	histoclear	1 hour		120
9	histoclear	1 hour		120
10	histoclear	1 hour, 15 min		140
wax1	paraplast	1 hour	62°C	140
wax2	paraplast	1 hour	62°C	140
wax3	paraplast	3 hours	62°C	140

Make sure that the functions "stirrer", "recirculation" and "pressure-vacuum" are on.

The machine stops when samples are in the last wax step until you say "drain retorte". After taking samples out, immediately store at 65°C in the Leica EG1160 (tissue embedding station) or in a tank in the water bath.

Clean tray with routine cleaning cycle that ASP200 requires.

1.2 Pouring Molds

You can store your molds and samples in the preheated chamber / water bath. Also keep your forceps and the metal moulds at 65°C. In the case, you do not use the EG1160 you also would need a heating plate and already melted paraplast.
Label the plastic moulds with pencil before use.

Place the metal mould at the heat plate, fill in some wax and quickly transfer the tissue material into the moulds. Using the preheated needle orient and compact the material into the middle of the mould. Put the labeled plastic mould on top and, if necessary, fill up with more wax. Carefully move mold to the 4°C area (EG1160), respectively to a room temperature area and leave there until wax is no longer liquid. Keep in fridge for some hours, then remove metal mould and store wax blocks at 4°C.

2 Sectioning

We buy already coated slides from Leica (STAR FROST) but of course it is more expensive. You also can coat the slides with VectabondTM reagent for your experiments. We make the sections with a Leica microtome.

Adjust a heat plate to 42°C. Label the slides with a pencil. It's important for getting nice ribbons, that the edge of the tissue block are straight and square, otherwise you don't get ribbons, but just single sections (we always cut the edge with a scalpel, as well to get two ribbons on one slide).
Place the mould into the microtome and trim it in order to get as close to the tissue as possible (there is a TRIM option at the microtome which generates 30µm trim sections). After trimming make 10 µm sections.
Take sections (we usually take about 5cm ribbons) and float them in water for about one min to remove the compressions. Fish two ribbons to one labeled slide (it is important to avoid air bubbles, otherwise the ribbons won't stick to the slides and the tissues will fall off during the experiment), remove the water by tapping the slide edge on a paper and place the slide back on the heat plate. Bake them overnight at 42°C.
Slides can be stored in a box at 4°C for a long time (1 year is no problem).

3 Probe Synthesis

The probe that you want to use needs to be cloned in a vector with RNA polymerase promoter (T7, T3, SP6) and subsequently gets linearized with enzymes which generate 5' overhang.
The other way is to generate the template by making PCR with suited primers containing the T7 promoter sequence (for sense probe the T7 promoter resides in the forward primer, for antisense probe it resides in the reverse primer).

Test the template concentration and use about 1µg in a transcription reaction.

3.1 Transcription Reaction

Set up the following reaction:	total 20 µl
sterile H_2O bidest	x µl
Linearized template 1µg	x µl
10 x RNA reaction buffer	2.0 µl
20mM $MgCl_2$	2.0 µl
10x NTP Mix (DIG labeling mix)	2.0 µl
RNAsin 20U (RNase inhibitor, 40U/µl Promega)	0.5 µl
RNA polymerase 40U	x µl

Incubate for max. 1h at 37°C (T7/T3 RNA polymerases) and 40°C (SP6 RNA polymerase).
We noticed that if you incubate 2h and longer, afterwards you can't digest the template anymore, which again may influence the hybridization in a negative way.
As a control remove 1.5 µl to load on a 1% agarose gel, to see, whether the transcription reaction worked.

Template-Digestion:
Add 2 units of RNase free DNase.
Incubate for 15 to 20 min at 37°C.
Keep at ice and stop the reaction by adding EDTA to a final concentration of 20 mM.

Add following solutions to precipitate the probe:
2.5 µl of 4M LiCl (final concentration 400mM)
75 µl of 100% EtOH p.a. (3-fold volume)

Mix well and incubate at -20°C over night or at least for 2h at -80°C.

Spin at 4°C for 30 min. You will see a pellet.
Wash the pellet with cold 70% Ethanol p.a. (750 µl).

Air-dry and resuspend in 50 µl of sterile water bidest.
Remove 3 µl to check on gel.

3.2 Probe Hydrolysis

We only hydrolyze the probe when it is >500 bp. The hydrolysis allows a better penetrance of the probe.

Set up the following reaction:
50 µl of RNA sample
50 µl of sterile water bidest
100 µl of 2x Hydrolysis Carbonate Buffer (200 mM Sodium Carbonate, pH 10.2)

Mix and incubate at 60°C for x time. We calculate the time using the following formula:

$$\textbf{Time} = \frac{\textbf{Li - Lf}}{\textbf{K Li Lf}}$$

Li: Initial length of the probe in kb
Lf: Final length of the probe in kb. We used Lf=0.2kb
K: 0.11Kb/min

After the incubation neutralize with 20 µl of 10% Acetic Acid. It is important to not hydrolyze longer, otherwise the probe will be too small.

Set the following reaction to precipitate the probe:
20 µl of 3M Sodium Acetat pH 5.2
1 µl of 20mg/ml glycogen, RNA grade
600 µl of 100% EtOH p.a.
Mix well and incubate at -20°C overnight.

Centrifuge for 30 min at 4°C.
Wash with ice cold 70% Ethanol p.a. (750 µl).

Air dry and resuspend in 50 µl of sterile H_20 bidest.
Remove 5 µl of sample to check in the gel (hydrolyzed RNA sample will smear between 100-400bp).

Dilute 1/10 with Hybridization Buffer and store at -20°C (1 µl of RNasin could be added to avoid any degradation for long term storage).

3.3 How much probe to use in a reaction

We usually determine the amount of probe to be used by doing pilot experiments. We usually use 5, 10, 15, 20, 40 µl of the probe (out of ~450 µl) in 125µl of Hybridization buffer to determine the best concentration to be used in the experiment.

To estimate the amount of labeled RNA, a dot blot can also be performed:

Solutions needed (see also immunological detection):

- Nylon N+ membrane
- +/- control (labeled control RNA / Hybridization buffer)
- 1xTBS
- Blocking solution (10% skim milk in 1xTBS)
- Anti-DIG antibody
- TBS
- TNM-50
- Western Blue

Spot 1μl of RNA dilutions on membrane (we normally do undiluted, 1:10, 1:100).
Crosslink the probes by drying at 52°C for 30 min.

Block for 30 min in Blocking solution (shaking).

Dilute the antibody 1:2000 in fresh Blocking solution and shake the membrane for about 1 hour.

Wash about 5x shortly with TBS.

Wash 2x shortly with TNM-50.

Apply Western Blue solution until signal is visible (about 10min).

Wash membrane with water and let it dry.

4 Hybridization

We do not DEPC treat the solutions or bake the dishes. We keep them separately and try to keep them free of RNase. But we autoclave all solutions (including water).

equipment you need:
- stirrer, water bath
- tips and pipettes (for 1 µl up to 1 ml)
- sterile 25, 250, 500 and 1000 ml cylinders with magnetic stirrer
- 3 sterile 1 liter bottles with magnetic stirrer
- 10 L H_2O bidest

4.1 Dewaxing and Rehydration

Dewaxing
- 2x Histoclear for each 10 min
- 2x 100% Ethanol (denatured is good enough) for each 2 min

Rehydration

Treat the slides about 1min each by dipping up and down till the streaks go away in the following solutions.
During preparation of the Ethanol series (denatured is good enough) first dilute NaCL with water then add with EtOH:
- 95% EtOH
- 90% EtOH
- 80% EtOH + 150 mM NaCl (7.5ml 5M NaCl in 250ml)
- 60% EtOH + 150 mM NaCl
- 30% EtOH + 150 mM NaCl

- 150 mM NaCl for 2 min
- 1x PBS for 2 min

The ethanol series can be used for the dehydration series too (see down) and also for about two more rounds of in situ.

4.2 Proteinase K digestion

This step is done to allow a better penetration of the probe into the tissue. The concentration of proteinase K is critical, in that it should be sufficient enough to allow better penetration and not high enough to destroy the morphology of the tissue.

Add proteinaseK to the prewarmed proteinaseK buffer to achieve a final concentration from 1 to 10 µg/ml (probe depending), stirring with the pipette tip to mix. Incubate the slides for 30 min. It is an important step: the time of the incubation should not be extended.

Stop the proteinase K digestion by keeping the slides for 2 min in Glycine-PBS and shake slowly. Wash for 2 min in 1x PBS.

4.3 Post Fixation

Keep in freshly prepared fixative for 5 min:
100 ml ethanol p.a.,
10 ml glacial acetic acid,
20 ml formaldehyde 37%
70 ml H_2O bidest

Discard the solution in the toxic waste (halogen free solvents).

Wash off the fixative for 2 min in 1x PBS.
If you do not accomplish the Acetylation step, wash off the fixative for 2x 5 min in 1x PBS and another 5 min in 150 mM NaCl.

4.4 Acetylation (optional)

The acetylation solution should already have been prepared. Put the solution on a stirrer, add 0.75ml Acetic anhydride, mix 4 to 5 sec and pour it over the slides (which already are in a appropriate box). Incubate for 2 min. Discard the solution in the toxic waste (halogen free solvents).

Wash 2x 5 min in 1x PBS and another 5 min in 150 mM NaCl.

4.5 Dehydration

Process the slides in the following solutions for about 30 sec each by dipping up and down (the same solution as the rehydration step):

- 150 mM NaCl
- 30% Ethanol + 150 mM NaCl
- 60% Ethanol + 150 mM NaCl
- 80% Ethanol + 150 mM NaCl
- 90% Ethanol
- 95% Ethanol
- 100% Ethanol

The dehydration steps will allow a better penetrance of the probes.
Store the slides in a closed box with a paper at the bottom soaked with few drops of ethanol at 4°C for several hours or process immediately. Before applying the probe dry the slides at room temperature for 30–60 min covered with a lid (to avoid contamination).

4.6 Hybridization

Prepare the humidified box (max capacity: 20 slides/box): we used plastic boxes and add 2-3 sheets of kitchen paper on the bottom. We stick plastic pipettes on the bottom to elevate the slides. We soak the kitchen paper with soaking solution. Put the box at 52°C to pre-incubate. Decide which probe to be used on which slide and accordingly prepare enough probe mixed with Hybridization Buffer (we usually make about 125 µl per slide).

Mix the probe with Hybridization Buffer and heat at 80°C for 2 min, chill on ice and keep on ice. Take at least 125 µl of probe and apply on one end of the slide; use a strip of parafilm to slowly and carefully cover the entire slide without air bubbles. If there are too many bubbles start all over again. Be carefully to avoid cross contamination between the probes (sense and anti-sense).

Incubate in the humidified box at 52°C overnight.

Also preheat 250 ml 2x SSC, 1 Liter 0,2x SSC and as well glass boxes in the 52°C incubator to start directly next day morning.

4.7 Washes

We have eliminated the RNase step in the washes.
Remove the parafilm of the slides by carefully dipping up and down for a few times in preheated 2x SSC and transfer the slides in preheated 0.2x SSC. Incubate at 52°C for 30 min. Change 0.2x SSC buffer 4 times for a total wash to at least 2 h. We usually don't shake the slides during the wash steps but one can do it.

Wash the slides in 0.2x SSC for 5 min at 37°C and repeat wash with 0.2x SSC at room temperature.
Finally put the slides in 1x PBS. The slides can be stored overnight. We usually proceed with detection on the same day.

5 Immunological Detection

5.1 Blocking

Put the slides in a box with blocking solution and incubate with gentle agitation at room temperature for 45-60 min. Don't discard the solution – you can use it for the first washing step after antibody binding.

5.2 Antibody binding

Prepare the antibody solution 10 min before applying. We usually dilute the antibody (Anti-Digoxigenin-AP Fab fragments) to a final concentration of 1:1250 in blocking solution (600 mU/ml).
Dry slides underneath, apply 100 µl of antibody solution, cover with a strip of parafilm and incubate at room temperature for 1 h and 30 min.

5.3 Washing antibody

Carefully remove the parafilm by dipping up and down in blocking solution and wash at room temperature for 10 min with gentle agitation.
Wash 3 times for each 30 min in TBS buffer.

5.4 Equilibration

Wash the slides twice in TNM buffer for each 5 - 15 min.

5.5 Color Reaction

We use the stabilized substrate solution from Promega called Western Blue. We filtrate the solution with a fluted filter before using to reduce particle dust on the slide.
(Optional: We add Levamisole to a final concentration of 1 mM, which is an inhibitor of alkaline phosphatase. But it is probe dependant. If the signal is weak, but specific and does not give background, do not use it. Levamisole will inhibit the endogenous alkaline phosphatase and therefore reduce the background. But of course, it will as well inhibit the alkaline phosphatase from the RNA probe. Therefore, it slows down the reaction.)
Dry the slides underneath, apply 100 µl onto a slide, cover with a strip of parafilm and place it in the humidified box. Wrap the box in aluminum foil and incubate overnight or longer at RT. You will have to monitor the color reaction under a microscope.

Put slides in a rack and let the parafilm float off in TNM buffer. Cover the section with a cover slip to check out under the microscope whether the color reaction is complete.
If the reaction is too weak, you can remove the cover slip (carefully), apply new substrate and leave for a longer time.
The time of the color reaction is specific to each probe and can vary from overnight to several days.

If the color reaction is complete, stop the reaction by rinsing in water or TE buffer.

6 Mounting

We usually wash the sections in TE buffer for at least 5 min and then mount in Mowiol 4-88. One can also keep in TE buffer for one or two days until mounting. Can be handy if the color reactions are very variable.

7 Solutions

Blocking Solution

Composition:	for 250 ml	stock solution:
1% Blocking reagent	2.5 g	
50 mM Tris pH7.5	12.5 ml	1 M Tris pH 7.5
150 mM NaCl	7.5 ml	5 M NaCl
0.3% TritonX100	0.75 ml	
Increase volume to	250 ml	H_2O bidest

Always make fresh prior to use.

50 x Denhardts

0.1% DEPC-H_2O

Add 1ml of diethyl pyrocarbonate (DEPC) in 1 Liter dH_2O.
Mix on a stirrer for at least 30 min to overnight.
Autoclave and store at room temperature.

50% Dextran sulfate

25 g of dextran sulfate in a total volume of 50 ml in sterile DEPC-H_2O.
Let it solve in a 60°C oven and stir overnight to dissolve.
Aliquot in 2 ml aliquots and store at -20°C.

0.5 M EDTA pH 8

Dissolve 46.53 g of EDTA disodium salt dihydrate (MW 372.24 g/mol) in a total volume of 250 ml dH_2O bidest.
Adjust pH to 8 with NaOH pellets (around 5-6g), before EDTA is not soluble.
Autoclave and store at room temperature.

Glycine-PBS

2 mg/ml Glycine in 1x PBS
Dissolve 0.5 g glycine in 250 ml sterile 1x PBS.
Always make fresh prior to use.

Hybridization Buffer

Composition:	for 10 ml
Formamide	5 ml
50% Dextran sulfate	2 ml
10x Hybridization Salts	1 ml
50x Denhardts	0.2 ml
50 mg/ml tRNA	0.1 ml
sterile DEPC-H_20	1.7 ml

Store at 4˚C up to 4 weeks.

10x Hybridization salts (From Drew protocol)

Composition:	for 20 ml	stock solution:
100 mM Tris pH 7.5	2 ml	1 M Tris pH 7.5
10 mM EDTA	0.4 ml	0.5 M EDTA pH 8
3 M NaCl	12 ml	5 M NaCl
Increase volume to	20 ml	H_2O bidest

Autoclave, aliquot and store at -20°C.

2x Hydrolysis Carbonate Buffer

Composition:	for 50 ml	
120 mM Na_2CO_3 (MW 105.99 g/mol)	0.64 g	
80 mM $NaHCO_3$ (MW 84.01 g/mol)	0.34 g	
Increase volume to	50 ml	H_2O bidest

Adjust pH 10.2 with 4-5 drops 5 M NaOH.
Autoclave, aliquot and store at -20°C.

10x Hydrolysis Neutralization Buffer

Composition:	for 20 ml	
10% Acetic Acid	2 ml	
Increase volume to	20 ml	H_2O bidest

Aliquot and store at -20°C.

1M Levamisole

Dissolve 2.408 g levamisole (MW 240.8 g/mol) in 10ml sterile DEPC-H_2O.
Store at room temperature enwrapped in aluminum foil.

4 M Lithium Chloride
Dissolve 8.48 g of lithium chloride (MW 42.39 g/mol) in 50 ml H_2O bidest.
Autoclave and store at room temperature.

1 M Magnesium Chloride
Dissolve 20.33 g of $MgCl_2 \times 6H_2O$ (MW 203.3 g/mol) in 100 ml H_2O bidest.
Autoclave and store at room temperature.

Mowiol 4-88
Dissolve 2.4 g of Mowiol in 6 ml Glycerin and 6 ml H_2O bidest. Mix for 1 h at room temperature. Add 12 ml 0.2M Tris-HCl (pH 8.5) and incubate at 50°C for 2 h (shaking). In the majority of cases the Mowiol doesn't solve completely – centrifuge for 15 min at 3500 rpm and aliquot the supernatant á 1 ml.
Store at -20°C.

5 M NaCl
Dissolve 292.2 g of NaCl (MW 58.44 g/mol) in 1000 ml H_2O bidest.
Autoclave and store at room temperature.

10x PBS

Composition:	for 1000 ml	
1.3 M NaCl (MW = 58,44 g/mol)	76.0 g	
30 mM NaH_2PO_4 (MW = 119.98 g/mol)	3.6 g	
70 mM Na_2HPO_4 (MW = 141.96 g/mol)	9.9 g	
Increase volume to	1000 ml	H_2O bidest

Adjust pH 7.0 with NaOH.
Autoclave and store at room temperature.

1x PBS
Mix 100ml 10x PBS with 900 ml sterile H_2O bidest.

Proteinase K
Dissolve the proteinase K to a final concentration of 10 mg/ml in sterile H_2O bidest.
Aliquot the solution in 50 µl aliquots and store at -20°C.

Proteinase K Buffer

Composition:	for 250 ml	stock solution:
100 mM Tris pH 7.5	25 ml	1 M Tris pH 7.5
50 mM EDTA	25 ml	0.5 M EDTA pH 8.0
Increase volume to	250 ml	sterile H_2O bidest

Adjust to pH 7.5 with conc. HCl.
Always prepare fresh prior to use.

20x SSC

Composition:	for 250 ml:	
3 M NaCl (MW = 58,44 g/mol)	43.83 g	
0.3 M Na_3-citrate x $2H_20$ (MW = 294,10 g/mol)	22.05 g	
Increase volume to	250 ml	H_2O bidest

Adjust to pH 7 with HCl.
Autoclave and store at room temperature.

Soaking Solution

Composition:	for one box:
20x SSC	3.5 ml
Formamide (the cheap one from Fluka!)	17.5 ml
Increase volume to	35 ml sterile H_2O bidest

Always prepare fresh prior to use.

3M Sodium Acetate

Dissolve 4.9 g of $C_2H_3O_2Na$ Sodium Acetate (MW 82.03 g/mol) in 20 ml H_2O bidest.
Adjust pH 5.2 with NaOH.
Autoclave, aliquot and store at -20°C.

TBS Buffer

Composition:	for 600 ml	
50 mM Tris pH7.5	30 ml	1 M Tris pH 7.5
150 mM NaCl	18 ml	5 M NaCl
Increase volume to	600 ml	sterile H_2O bidest

Always make fresh prior to use.

TE Buffer

Composition:	for 250 ml	stock solution:
10 mM Tris pH 8	2.5 ml	1 M Tris pH 8
1 mM EDTA pH 8	0.5 ml	0.5 M EDTA pH 8
Increase volume to	250 ml	H_2O bidest

Autoclave and store at room temperature.

TNM Buffer

Composition:	for 500 ml	stock solution:
100 mM Tris pH 9.5	50 ml	1 M Tris pH 9.5
100 mM NaCl	10 ml	5 M NaCl
50 mM $MgCl_2$	25 ml	1 M $MgCl_2$
Increase volume to	500 ml	sterile H_2O bidest

Adjust pH to 9.5.
Always prepare fresh prior to use.

1 M Tris pH 7.5 / pH 8.0 / pH 9.5

Dissolve 60.57 g of Tris Base (MW 121.14 g/mol) in 400 ml H_2O bidest.
Adjust pH to 7.5 / 8.0 / 9.5 with a lot of conc. HCl.
Increase the volume to 500 ml with H_2O bidest.
Autoclave and store at room temperature.

t-RNA from E.coli MRE600 50 mg/ml

Dissolve 100 mg in 2 ml of sterile DEPC-H_2O (directly in the supplied vial).
Aliquot and store at –20˚C.

8 Chemicals/equipment/material

Acetic Acid
Sigma A6283

Acetic Anhydride
Sigma A6404

Anti-Digoxigenin-AP Fab fragments (150U/200µl)
Roche 11 093 274 910

Blocking Reagent
Roche 11 096 176 001

Coated Slides
STAR FROST Knittel-Glaeser
Adhesive slide 90° white, Leica Biosystems Nussloch GmbH 14071239141

50 X Denhart's Solution
Sigma Aldrich D2532

Dextran sulfate 500 Natriumsalz
Applichem A4970

DIG RNA Labeling Mix
Roche 11 277 073 910

DNAse I, RNAse-free (10000 U)
Roche 04 716 728 001

EDTA
Sigma E5134

Formaldehyde 37%
Sigma 252549

Formamide
deionized for Hybridization Buffer: Applichem A2156
for Soaking Solution: Sigma 47671

Glass vials
Zefa Z.0421006

Glycerol
Sigma G5516

Glycogen RNA Grade
Fermentas R0551

Glycine
Sigma G7126

Histoclear
Vogel ND-HS-2001

Levamisole Hypochloride
Applichem AA4341,0005

Lithium chloride
Sigma L9650

Magnesium Chloride
Fluka 63068

Microtome
Leica RM 2145 (Kat.-Nr. 050131379)

Moulds
disposable plastic: Jet I Einbettkassetten - Leica Histoservice
metal: Klinipath B.V. P.O. Box 195 6920 Ad Duiven Netherlands
www.klinipath.nl
Base molds 15*15*5 mm No 3051

Mowiol 4-88
Roth 0713

Paraplast Embedding Media (Paraplast Plus)
Sigma P3683

Proteinase K from Tritirachium album
Sigma P2308

RNasin Plus RNase inhibitor
Promega N2611

SP6 RNA polymerase 2000 U
NEB M0207S

T7 RNA polymerase 5000 U
NEB M0251S

Sodium acetate
Sigma S2889

Sodium bicarbonate ($NaHCO_3$)
Fluka 71628

Sodium carbonate (Na_2CO_3)
Merck 1.06392.1000

Sodium chloride
Applichem A1371

Sodium citrate tribasic Dihydrate ($C_6H_5Na_3O_7 \cdot 2H_2O$)
Fluka 71405

Sodium dihydrogen phosphate (NaH_2PO_4)
Fluka 71496

Di-Sodium hydrogen phosphate (Na_2HPO_4)
Fluka 71640

Sodium hydroxide Pellets
Fluka 71690

Triethanolamine
Sigma T1377

t-RNA, RNase free
Roche 10 109 541 001

Triton X-100
Applichem A4975

Western Blue
Promega S3841