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Detecting Spaciotemporal Transcript Accumulation in Maize by RNA *In Situ* Hybridization

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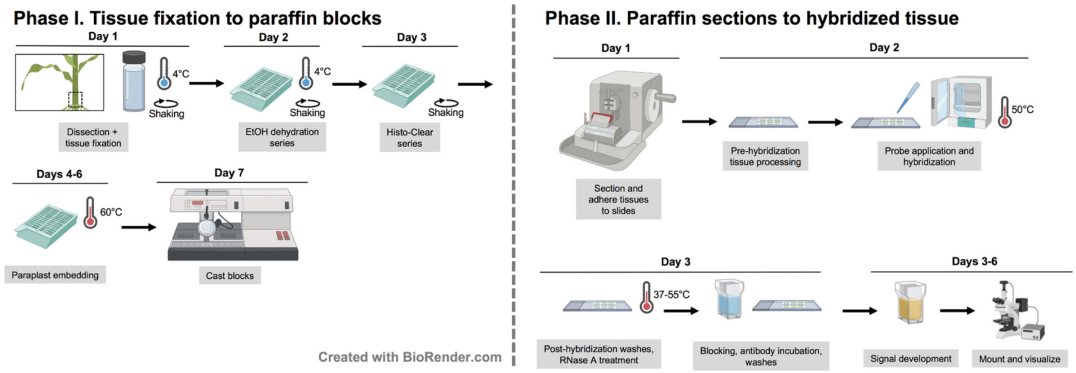
[Abstract] RNA *in situ* hybridization is a method for visualizing spatiotemporal transcript accumulation in cells and tissues. The method provides clear resolution, is highly sensitive and specific, and can uncover gradients of transcript accumulation within a histologically-intact tissue, which is not possible currently with other methods for transcript detection. RNA *in situ* hybridization, however, is not a quantitative approach for gene expression. Protocols for RNA *in situ* hybridization have numerous steps that can span several days of work, complicating troubleshooting procedures. Here, we build on previously published RNA *in situ* hybridization protocols optimized for paraffin-embedded and sectioned maize tissue (Jackson, 1991; Long *et al.*, 1996; Javelle *et al.*, 2011) by providing additional measures for optimized transcript detection.

Graphic abstract:



Workflow for RNA in situ hybridization

Keywords: Maize, Gene Expression Analysis, mRNA Detection, *In Situ* Hybridization

[Background] Differential gene expression in time and space allows cells possessing the same genetic material to take on different identities. Indeed, such shifts in gene expression are often responsible for driving evolutionary changes in patterning or morphology across organisms (Carroll, 2005). Therefore, insights into developmental processes are enhanced by observations of gene expression in its native

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context within a tissue. Approaches such as RT-qPCR and RNA-Seq rely on the destructive processing of tissues to extract RNA for gene expression analysis. RNA *in situ* hybridization offers the advantage of providing spatially and temporally resolved analyses of gene expression within the native tissue and cell type context. In maize and other plant species, RNA *in situ* hybridization has been used to probe gene function, tissue patterning, the evolutionary modification of gene expression, and corroborate single-cell expression data (Jackson *et al.*, 1994; Whipple *et al.*, 2010; Johnston *et al.*, 2014; Strable *et al.*, 2017; Knauer *et al.*, 2019; Satterlee *et al.*, 2020). Here, we present a method for detecting the accumulation of transcripts by RNA *in situ* hybridization in maize, a model genetic system and important staple crop. This protocol demonstrates the technique in shoot apex tissue sections where new leaf and stem tissue is initiated from pluripotent cell populations within the shoot apical meristem. Hybridization of an antisense antigen-tagged RNA probe to complementary RNAs within the tissue enables the qualitative detection of transcript accumulation patterns by an enzyme-conjugated antibody that catalyzes a colorimetric staining reaction. This protocol is readily adaptable to other maize tissues and is based on Jackson (1991) and Javelle and coworkers (2011).

Materials and Reagents

A. Consumables
   1. Paper towels
   2. Kimwipes
   3. Razor blades (single edge, steel)
   4. Pencil
   5. Parafilm
   6. Adhesive Glass Microscope Slides (VWR, VistaVision™ HistoBond®, catalog number: 16004-406)
   7. Cover slips (25 × 60 mm; VWR, catalog number: 89082-272)
   8. Glass Pasteur pipettes
   9. Glass vials with caps (VWR, catalog number: 66012-022)
   10. Paraplast Plus® (McCormick Scientific, catalog number: 15159-464)
   11. Cups with lids (VWR, catalog number: 89508-714)
   12. Biopsy cassettes (VWR, catalog number: 25608-756)
   13. Disposable base molds (VWR, catalog number: 100501-996)
   14. Embedding ring (VWR, catalog number: 87002-374)

B. Biological material
   Maize tissue (*e.g.*, shoot apex, inflorescence, root)

C. Reagents
   1. Nuclease free water

2. Ethanol (VWR, catalog number: EM-EX0276-3S)
3. Histo-Clear II (VWR, catalog number: 101412-882)
4. Acetic acid glacial (VWR, catalog number: BDH3094-2.5LG)
5. 37% Formaldehyde solution (VWR, catalog number: 97064-604)
6. Permount (Fisher, catalog number: SP15-100)
7. Ethanol series: dilutions to 95%, 85, 70, 50 in distilled water
8. Eosin Y (VWR, catalog number: 97061-034)
9. TOPO™ Cloning Kit for Sequencing (Thermo Fisher Scientific, catalog number: K4575-01) or pGEM®-T Easy Vector System (Promega, catalog number: A1380)
10. RNase OUT; 40 U/µl (ThermoFisher, catalog number: 10777019)
11. RQ1 RNase-Free DNase; 1 U/µl (Promega, catalog number: M6101)
12. DIG RNA labeling mix (Roche, catalog number: 11277073910)
13. SP6 RNA polymerase 1,000 U (Roche, catalog number: 10810274001)
14. T3 RNA polymerase 1,000 U (Roche, catalog number: 11031163001)
15. T7 RNA polymerase 1,000 U (Roche, catalog number: 10881767001)
16. RNase-A (Roche, catalog number: 10109142001)
17. Blocking Reagent (Roche, catalog number: 11096176001)
18. Anti-Digoxygenin-AP, Fab fragments from sheep, 150 U (Roche, catalog number: 11093274910)
19. NBT/BCIP stock solution (Roche, catalog number: 11681451001)
20. Mini Quick Spin RNA columns (Sigma, catalog number: 11814397001)
21. QIAprep Spin Miniprep Kit (Qiagen, catalog number: 27104)
22. DNA clean & concentrator kit (Zymo Research, catalog number: D4033)
23. tRNA from *E. coli* (Roche, catalog number: 10109541001)
24. Triton® X-100 (Sigma, catalog number: X100)
25. Deionized formamide (Sigma, catalog number: S4117)
26. Protease from Streptomyces griseus Type XIV (Sigma, catalog number: P5147)
27. Triethanolamine-HCl (Sigma, catalog number: T1502)
28. Acetic anhydride (Sigma, catalog number: 320102)
29. Dextran sulfate 50% solution (Sigma, catalog number: S4030)
30. Denhardt's solution 50× concentrate (ThermoFisher, catalog number: 750018)
31. Albumin from bovine serum ≥98% (Sigma, catalog number: A9418)
32. TAE-agarose gel
33. Glycine
34. 1 M Tris-HCl (at pH 7.5, 8.0, and 9.5)
35. 0.5 M EDTA
36. 5 M NaCl
37. FAA fixative (see Recipes)
38. 1× PBS buffer (see Recipes)
39. 2× carbonate buffer (see Recipes)

40. 10% Glacial Acetic Acid (see Recipes)
41. 3 M NaOAc pH 5.2 (see Recipes)
42. 4 M NH_4OAc (see Recipes)
43. 1× TBS buffer (see Recipes)
44. 1× TN buffer (see Recipes)
45. Protease buffer (see Recipes)
46. 1× *in situ* hybridization salts (see Recipes)
47. 0.2× SSC (see Recipes)
48. 1× NTE buffer (see Recipes)

Equipment

1. Pipettes
2. Forceps (VWR, catalog number: 82027-386)
3. Slide warmer (set to 37-42 °C)
4. Oven set at 42 °C
5. Vacuum pump
6. Microtome
7. Fine paintbrushes (*e.g.*, Virtuous Arts Fine Paintbrushes, amazon.com)
8. Stainless steel slide rack with handle (VWR, catalog number: 25461-014)
9. Tupperware (*e.g.*, medium rectangular 15.5 × 8.1 × 7.3 cm of volume 412 ml)
10. Large Tupperware with sealable lids
11. Glass staining dish (VWR, catalog number: 25461-016) with cover (VWR, catalog number: 25461-018)
12. Orbital shaker
13. Heating block
14. Incubator up to 60 °C capacity
15. Ovens and water baths for 37 °C and 55 °C
16. Centrifuge at room temp and 4 °C
17. Fume hood
18. Gel electrophoresis setup
19. Microscope equipped with a camera

Procedure

A. Fixation of maize material

Note: Procedures A through C are the same as those outlined in Strable et al., 2020 for toluidine blue staining of paraffin-sectioned maize tissue.

1. Prepare fresh FAA fixative (Recipe 1) and place on ice. Formaldehyde is toxic; therefore, FAA

solution should be prepared and used in a fume hood and disposed of properly.

2. Harvest maize tissue samples and place immediately in ~20 ml fresh FAA fixative in glass vials on ice.
3. Apply a vacuum (~500 mm Hg) to the glass vials with samples in FAA on ice. Maintain vacuum for 15-20 min; effervescence released from the tissue should be obvious, but fixative should not boil.
4. Release vacuum gently (over a minute) and renew the FAA fixative.
5. Keep the glass vials with samples in FAA overnight at 4 °C shaking at 50-70 rpm.

B. Embedding fixed maize material

1. Pre-cool the ethanol series (Dilutions to 95%, 85%, 70%, 50% in distilled water) on ice or at 4 °C in plastic cups.
2. Transfer samples from glass vials to biopsy cassettes to facilitate moving samples through the following ethanol and Histo-Clear series.
   Note: If biopsy cassettes are not used, decant and replace the liquid through each series while leaving the specimens in the glass vial.
3. Dehydrate samples by moving the biopsy cassettes with specimens through a graded ethanol series in lidded cups on ice, shaking on an orbital shaker at 90 rpm:
   50% EtOH for 1 h.
   70% EtOH for 1 h.
   85% EtOH for 1 h.
   95% EtOH for 1 h.
   100% EtOH #1 for 1 h.
   100% EtOH #2 for 1 h.
   100% EtOH #3 for 1 h.
   100% EtOH with 0.01% (w/v) Eosin overnight at overnight at 4 °C.
   Note: Any volume is sufficient as long as it covers the specimens. The plastic cups hold 100 ml. EtOH solutions can be reused and stored at room temp. EtOH solutions can be reused and stored at room temp for a few experiments if properly sealed and remain unevaporated.
4. Transfer samples to fresh 100% EtOH at room temperature for 1 h.
5. Replace ethanol with Histo-Clear II through the following graded series in lidded cups at room temperature, shaking on an orbital shaker at 90 rpm:
   EtOH to Histo-Clear (3:1 v/v) for 1 h.
   EtOH to Histo-Clear (1:1 v/v) for 1 h.
   EtOH to Histo-Clear (1:3 v/v) for 1 h.
   100% Histo-Clear #1 for 1 h.
   100% Histo-Clear #2 for 1 h.
   100% Histo-Clear #3 for 1 h.
   100% Histo-Clear #4 for 1 h.

6. Add 0.25 vol of Paraplast Plus® chips to the samples in 100% Histo-Clear #4 and place at 42 °C oven overnight.
7. Add Paraplast Plus® chips to several cups and place in a 60 °C to ensure fresh molten wax is available for subsequent wax changes.
8. Replace molten Paraplast Plus®, kept at 60 °C, at least 6 times by decanting molten Paraplast Plus® from samples and replacing with fresh molten Paraplast Plus®. This can be accomplished 3 times every 4 h over 2 days, or once a day for 6 days.
9. Final embedding of tissue for sectioning will vary depending on the tissue type and desired plane of section. Disposable plastic molds and rings are useful to maintain correct orientation of tissue. Mounted, embedded samples are stable for a year or more at 4 °C.

C. Microtome sectioning of embedded maize material
1. Warm embedded blocks to room temperature.
2. Trim the block into a trapezoidal shape (see Figure 1) using a razor blade, leaving a ~1 mm perimeter of wax around the tissue.

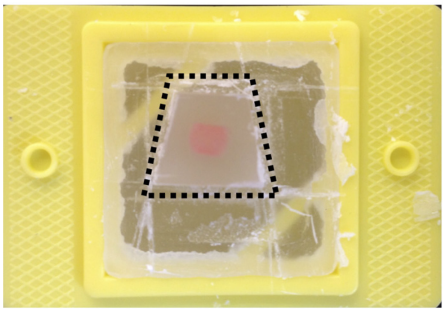

Figure 1. Trapezoid shape in a paraffin block. Image of trapezoid shape trimmed into paraffin block around embedded tissue stained pink with Eosin.

3. Mount tissue block onto the block holder of the microtome by positioning the longer of the parallel edges of the trapezoid at the bottom (closet to the microtome blade).
4. Section to a thickness of 8-10 µm.
5. Use fine paintbrushes to move wax ribbons to a bench lined with parafilm, which will allow selecting tissue sections of interest by directly cutting with a razor blade.
6. Pipet 1 ml of MilliQ water at room temperature onto the surface of HistoBond glass microscope slide and carefully move the selected wax ribbon onto the water as to float the sections of interest on the slide.
7. Transfer the slide slowly to the slide warmer to heat the slide-water-wax ribbon combination. This method of heating helps to spread the ribbon on the surface of the water to achieve even adhering of the tissue to the surface of the slide.
8. Use a kimwipe to wick away water carefully to lower the ribbon evenly on the slide.

9. Allow slides to dry and tissue to adhere for a minimum of six hours to overnight at 37-42 °C on the slide warmer.
10. Transfer slides to metal slide rack. Slides with adhered sectioned tissue should be stored in an air-tight Tupperware with packets of silica desiccant for several days to a few weeks at 4 °C.

D. Probe preparation

General notes: *All steps for in vitro transcription of sense and antisense templates are sensitive to RNase activity. Some protocols require solutions to be made RNase-free with DEPC-treated water, glassware to be held at 180 °C for a minimum of 3 h, and plastic containers to be treated with 0.2 NaOH for 30 min and then rinsed in DEPC-treated water. In our hands, none of these procedures are essential, but we regularly use DEPC-treated water (0.5 ml/L, shaken, overnight at room temp. and autoclaved) and occasionally nuclease-free water. We store and maintain separate reagents, glassware, plasticware for in situ hybridization.*

1. Cloning

   Antisense RNA *in situ* probes can be synthesized from one or more targeted regions of a cDNA of interest. General rules of thumb for designer probes: 1) avoid highly repetitive sequences in the gene body; 2) transcription factor DNA binding motifs generally will produce specific hybridization signals; 3) consider each transcript is unique in terms of design and several probes per cDNA may be required; 4) targeted regions of the cDNA can vary considerably in length, from as short as 150 base pairs (bp) to no longer than 1,500 bp.

   a. PCR amplified cDNA (complete exons or exon fragments of known tissue/developmental/stressed/*etc.* expressed genes) templates for *in vitro* transcription (see Step D2) are cloned into suitable vectors that harbor T3, T7 or Sp6 promoters (*e.g.*, pCR™4-TOPO™ Vector [Thermo Fisher Scientific] or pGEM®-T Easy Vector System [Promega]).

   b. Alternatively, to avoid subcloning PCR fragments into a vector, T3, T7 or Sp6 promoter sequences can be incorporated into a 5'-tail on the reverse primer used for PCR amplification of probe region.

2. *In vitro* transcription of sense and antisense templates

   a. Digest 2 µg of plasmid DNA in a 100 µl digestion reaction with restriction enzymes that cut at the end of the insert opposite the promoter. In linearizing the plasmid, avoid restriction enzymes that produce a 3'-overhang, which can be used as a template for transcription and produce artifact transcripts.

      Note: *Alternatively, the probe region with T3, T7, or SP6 promoter sequences from Step D1b can be PCR amplified to generate a DNA template for in vitro transcription.*

   b. Post-digestion verify the plasmid has been linearized by analyzing a 5 µl aliquot with gel electrophoresis.

   c. Use a DNA clean & concentrator kit to purify the linearized plasmid.

   d. Set up the *in vitro* transcription reaction as follows:

1 µl purified DNA (1 µg/µl)
2 µl 10× transcription buffer
2 µl 10× DIG RNA labeling mix
1 µl RNase OUT
2 µl T3, T7 or SP6 polymerase (20 U/µl)
H_2O up to 20 µl

e. Mix and incubate at 37 °C for 2 h.
f. Check 1 µl of *in vitro* transcription product by gel electrophoresis.
g. Add 80 µl Nuclease-free H_2O, 1 µl DNase I (10 U/µl) and 1 µl tRNA (100 mg/ml) to the transcription reaction and incubate the reaction at 37 °C for 10 min to remove the DNA template. Optional: verify DNA removal by checking 5 µl with gel electrophoresis
h. Purify the *in vitro* transcription product:
   i. Add 95 µl (or equal volume as above) 4 M NH_4OAc.
   ii. Add 195 µl (or 2× volume as above) 100% ethanol.
   iii. Precipitate at -20 °C for 30 min.
   iv. Centrifuge at ~17,900 × *g* at 4 °C for 5 min.
   v. Rinse pellet in 70% ethanol.
   vi. Centrifuge at ~17,900 × *g* at 4 °C for 5 min.
   vii. Dry pellet at room temperature. Use caution not to over dry as the *in vitro* transcription product is RNA, which when dry is recalcitrant to resuspension.
   viii. Resuspend pellet in 100 µl nuclease-free H_2O

3. Probe hydrolysis: probes >250 nucleotides can be partially hydrolyzed into RNA fragments of 150 nucleotides that will more efficiently work in sectioned tissues.
   a. Add 100 µl (or equal volume) 2× carbonate buffer (80 mM $NaHCO_3$; 120 mM Na_2CO_3) to the resuspended purified *in vitro* transcription product.
   b. Incubate at 60 °C for incubation times empirically determined as follows:
      Incubation time = $(L_i - L_f)/(K \times L_i \times L_f)$
      where,
      L_i = initial probe length (in kilobases);
      L_f = final probe length (0.150 kb);
      K = 0.11 kb/min.
   c. Neutralize hydrolysis reaction by adding 10 µl (or $1/20^{th}$ volume) of 10% acetic acid.
   d. Purify the hydrolyzed probe:
      i. Add 21 µl (or $1/10^{th}$ volume as above) 3 M NaOAc pH 5.2.
      ii. Add 420 µl (or 2× volume as above) 100% ethanol.
      iii. Precipitate at -20 °C for 30 min.
      iv. Centrifuge at ~17,900 × *g* at 4 °C for 5 min.
      v. Rinse pellet in 70% ethanol.
      vi. Centrifuge at ~17,900 × *g* at 4 °C for 5 min.

vii. Dry pellet at room temperature.

viii. Resuspend pellet in 50-70 µl 50% deionized formamide.

4. Store the concentrated probe at -80 °C until use.

E. Tissue pretreatment

General notes: *All steps for in vitro transcription of sense and antisense templates are sensitive to RNase activity. We regularly use DEPC-treated water (0.5 ml/L, shaken, overnight at room temp. and autoclaved) and occasionally nuclease-free water. We store and maintain separate reagents, glassware, plasticware for in situ hybridization. Solution volume will depend on container size and the number of slides to be pretreated, but for medium rectangular Tupperware (15.5 × 8.1 × 7.3 cm), a volume of 350 ml is sufficient. Before starting pretreatments, prepare all solutions ahead of time including the 0.1 M Triethanolamine-HCl pH 8.0 buffer. Add the acetic anhydride solution to the triethanolamine buffer right before use (Step E11). Thaw protease stock at the time of use. All steps are carried out at room temperature unless otherwise noted.*

1. Prewarm 1× protease buffer (100 mM Tris-HCl pH 8.0; 50 mM EDTA pH 8.0) to 37 °C.
2. Transfer slide rack through the following series to deparaffinize and rehydrate tissue sections:
   100% Histo-Clear for 10 min in a glass staining dish.
   100% Histo-Clear for 10 min in a glass staining dish.
   100% EtOH #1 for 1 min.
   100% EtOH #1 for 1 min.
   95% EtOH for 30 s.
   85% EtOH for 30 s.
   70% EtOH for 30 s.
   50% EtOH for 30 s.
   DEPC/nuclease free water for 1 min.
   Note: *Save this series to be used again in Step E14.*
3. Transfer slide rack to 1× PBS for 2 min.
4. Add 625 µl protease (50 mg/ml) to 250 ml protease buffer prewarmed to 37 °C, stir, and transfer slide rack for 30 min at 37 °C.
   Note: *Adjust protease amount to buffer volume accordingly. Protease is necessary to digest proteins and increase probe access to mRNA in the cellular matrix. Incubation time can be optimized to balance achieving maximum hybridiation signal without destructive breakdown of tissue.*
5. Transfer the slide rack to 0.2% glycine dissolved in 1× PBS for 2 min to neutralize the protease activity.
6. Transfer the slide rack to 1× PBS for 2 min to rinse slides.
7. Transfer the slide rack to 1× PBS for 2 min.
8. Transfer the slide rack to 4% formaldehyde solution in 1× PBS for 10 min to re-fix RNA after protease treatment.

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Note: *Formaldehyde is toxic, and the solution should be prepared in a fume hood and disposed of properly.*

9. Transfer the slide rack to 1× PBS for 2 min to rinse slides.
10. Transfer the slide rack to 1× PBS for 2 min.
11. Immediately prior transfer of slide rack to the triethanolamine buffer, add acetic anhydride at 0.5% (v/v) to the triethanolamine buffer in a glass dish, stirring constantly with stir bar.
12. Transfer the slide rack to the acetic anhydride-triethanolamine buffer, stirring constantly for 10 min. Because the acetic anhydride is unstable in triethanolamine buffer, this requires constant mixing of the acetic anhydride-triethanolamine buffer with the stir bar. The slide rack should be elevated in the glass dish above the stir bar (see Figure 2).
    Note: *This step acetylates positively charged amino groups that may lead to non-specific binding of the probe. Acetic anhydride is toxic, and the solution should be prepared in a fume hood and disposed of properly.*

Figure 2. Stirring apparatus for acetic anhydride-triethanolamine buffer. Image of a stir plate, glass Pyrex dish, lead ring flask weight (orange), and stir bar. The slide rack sits atop the flask weight while the stir bar mixes the buffer in a fume hood.

13. Transfer the slide rack to 1× PBS for 2 min.
14. Transfer the slide rack to 1× PBS for 2 min.
15. Transfer slide rack through the following series to dehydrate the tissue sections:
    DEPC/nuclease free water for 1 min.
    50% EtOH for 30 s.
    70% EtOH for 30 s.
    85% EtOH for 30 s.
    95% EtOH for 30 s.
    100% EtOH for 1 min.
    100% EtOH for 1 min.

16. Store rack of slides in an airtight container with a small volume of 100% ethanol at the bottom. The tissue is now ready for probe hybridization.
    Note: *Slides can be stored like this for a few hours before proceeding with hybridization.*

F. Probe hybridization
1. Prepare a heating block to 80 °C.
2. Prepare hybridization buffer.
   Note: *80 µl hybridization buffer (this step) is needed per slide along with 20 µl of diluted probe (Step F2), therefore the amount of hybridization buffer to prepare depends on the number of slides. It is advised that the hybridization buffer be prepared in a 10% excess to ensure adequate amounts for each probe. Dextran sulfate has high viscosity; preheating the solution to 60 °C aids in pipetting, or pipette tips can be cut to help with pipetting. The recipe here is for 1 slide.*
   Mix the following fresh:
   7 µl of nuclease free water
   1 µl tRNA (100 mg/ml)
   2 µl 50× Denhardt's solution
   40 µl deionized formamide
   10 µl 10× *in situ* hybridization salts
   20 µl 50% dextran sulfate
3. Prepare diluted probes
   a. For each slide, mix 1 µl of concentrated probe (from Step D4) with 19 µl 50% deionized formamide.
   b. Denature the probe by heating at 80 °C for 2 min.
   c. Immediately place on ice.
   d. Centrifuge to collect contents.
4. Prepare probe-hybridization mixture. For each slide, add 80 µl of hybridization buffer to 20 µl denatured and diluted probe. Mix well by gentle pipetting to avoid introducing air bubbles.
   Note: *Probe concentration should be determined empirically. We routinely try dilutions of 0.5-4 µl of concentrated probe per 100 µl of probe-hybridization mixture per slide.*
5. Air dry slides from Step E15 for 5-10 min on a clean surface.
6. A slide-sandwiching method is used for hybridization (see Figure 3).
   This requires two sample slides (or one sample slide plus a blank slide) that are incubated with the same probe. Pipet 100 µl of probe-hybridization mixture onto the upper right corner of each slide and gently bring them together (hence, sandwich.) Ensure the probe-hybridization mixture covers all tissue sections without any air bubbles. If air bubbles form, do not lift or separate the two slides as the adhesion between them and the probe-hybridization mixture will damage the tissue sections. Rather, pipette a small amount of probe-hybridization mixture between the slides in the area of the air bubble(s) or tap the slide sandwich to reposition/remove the air bubbles.

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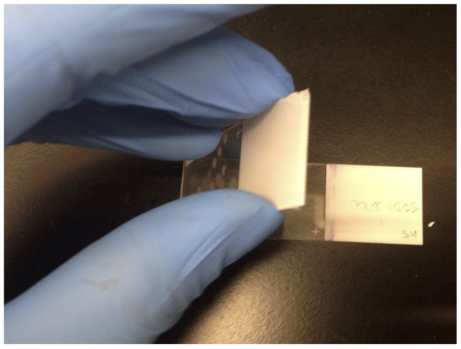

Figure 3. Preparing a slide sandwich after application of probe hybridization mix. After probe-hybridization mixture is applied to the upper right corner of each slide and spread evenly across the tissue sections, gently bring the slides together to form a sandwich.

An alternative to slide sandwiches: Pipette the probe-hybridization mixture onto the right upper corner of the slide and gently lower a coverslip onto the slide. Ensure the probe-hybridization mixture covers all tissue sections without any air bubbles. If air bubbles form, do not lift off the coverslip as the adhesion between it, the probe-hybridization mixture and the slide will damage the tissue sections; rather, tap the coverslip to reposition/remove the air bubbles.

7. Prepare a humidity chamber (see Figure 4).
   We use a lid-locking Tupperware container with an airtight lid. Cover the bottom of the container with paper towels and thoroughly moisten with nuclease free water. Place a clean Eppendorf tube rack in the container to serve as a surface for the slide sandwiches. Multiple slide sandwiches of the same probe can be abutted next one another, but slide sandwiches with different probes cannot, otherwise the probe-hybridization mixture will transfer by wicking and cause hybridization artifacts. Once all sides are done, seal the box tightly.

   For the coverslip method, use parafilm to cover the moistened paper towels leaving a margin of moist paper towel exposed. Place the slides on top of the parafilm. Once all sides are done, seal the box tightly.

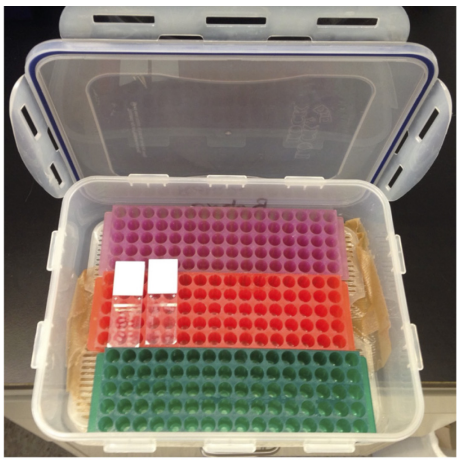

Figure 4. Humidity chamber for probe-tissue hybridization. A lid-locking Tupperware

container with an airtight lid with paper towels and thoroughly moisten with nuclease free water and clean Eppendorf tube racks as a surface for the slide sandwiches.

8. Place the container into a hybridization oven set to a temperature range of 50-55 °C for 16-20 h.
9. Prepare sufficient volumes of the following buffers for immediate use the next day: 1× SSC buffer at 55 °C; 1× NTE buffer at 37 °C. All other solutions can be prepared on the day of use.

G. Post-hybridization washing

1. Submerge slides in 55 °C 0.2× SSC, and gently separate the slides by sliding them horizontally across one another. Avoid pulling the slides apart vertically as the resulting suction can damage the adhered tissues. Place the separated slides in a slide rack submerged in 55 °C 0.2× SSC solution.
   Note: If a weak signal is observed at the end of the protocol, the concentration of SSC can be raised up to 1× to improve hybridized probe retention. If using a lower hybridization temperature, the temperature of SSC washes should be at least 5 °C higher than that used for hybridization.
2. Transfer the slide rack to fresh 55 °C 0.2× SSC and incubate while shaking gently at 55 °C for 1 h.
3. Prepare the BSA/Triton-X 100 and the Roche Blocking Solution during the incubation.
4. Repeat Step G2 with fresh 55 °C 0.2× SSC.
5. Transfer slides to 37 °C 1× NTE and incubate while shaking gently at 37 °C for 5 min.
6. Repeat Step G5 with fresh 37 °C 1× NTE.
7. Transfer slides to 37 °C 1× NTE supplemented with 20 µg/ml RNase A and incubate while shaking gently at 37 °C for 30 min. Use a designated container for this step to avoid RNase A contamination.
8. Transfer slides to 37 °C 1× NTE and incubate while shaking gently at 37 °C for 5 min.
9. Repeat Step G8 with fresh 37 °C 1× NTE.
10. Transfer the slide rack to fresh 55 °C 0.2× SSC and incubate while shaking gently at 55 °C for 1 h.

H. Antibody binding and washing

1. Transfer the slide rack to 1× TBS and incubate while shaking gently at room temperature for 5 min.
2. Transfer the slide rack to Roche Blocking Solution and incubate while shaking gently at room temperature for 45 min.
3. Transfer the slide rack to the BSA/Triton-X 100 solution and incubate while shaking gently at room temperature for 45 min.
4. Dilute 1:1,000 anti-digoxigenin (DIG), fab fragment antibody in the BSA/Triton-X 100 solution and mix gently by inversion. Add 5 ml of the mixed antibody solution to Copeland jars.

5. Sandwich the slides together and place them in a Copeland jar, allowing capillary action to draw up the antibody solution between the slides. Then, drain the sandwiched slides by applying a lint-free tissue to one side. Place the drained slides back in the Copeland jar.
6. Incubate the slides in the antibody solution for 2 h. at room temperature or overnight at 4 °C.
7. Submerge the sandwiched slides in BSA/Triton-X 100 solution, and gently separate the slides by sliding them horizontally across one another. Avoid pulling the slides apart vertically as the resulting suction can damage the adhered tissues. Place the separated slides in a slide rack submerged in BSA/Triton-X 100 solution.
8. Incubate slides in BSA/Triton-X 100 solution for 15 min. gently shaking at room temperature.
9. Repeat Step H8 three more times, refreshing the BSA/Triton-X 100 solution after each wash.

I. NBT/BCIP staining

1. Incubate slides in 1× TBS buffer for 5 min, gently shaking at room temperature.
2. Incubate slides in 1× TN buffer for 5 min, gently shaking at room temperature.
3. Repeat Step I2 with fresh 1× TN buffer.
4. Prepare NBT/BCIP staining solution (20 µl NBT/BCIP per ml TN buffer) and add 5 ml to each Copeland jar.
5. Sandwich the slides together and place them in a Copeland jar, allowing capillary action to draw up the NBT/BCIP solution between the slides. Then, drain the sandwiched slides by applying a lint-free tissue to one side. Place the drained slides back in the Copeland jar. NBT/BCIP is light sensitive, so place the incubating slides in a dark space.
6. The signal for highly expressed genes can appear within several h, while the signal for more lowly expressed genes can take longer than one day to appear. Color development typically ceases after 3 days, at which point undesired background signal may develop. The development process can be monitored by examining the sandwiched slides under a light microscope. Draining and refreshing the probe solution once a day can aid with signal development and reduce background signal.
7. When sufficient staining has developed (see Figure 5) submerge the slides in 1× TE buffer and gently separate by sliding them horizontally across one another. Avoid pulling the slides apart vertically as the resulting suction can damage the adhered tissues. Place the separated slides in a slide rack submerged in 1× TE buffer and incubate for 5 min at room temperature.
8. Repeat the incubation from Step I7 with fresh 1× TE buffer.

J. Dehydration and coverslip mounting

1. Transfer the slides to water for 1 min, then proceed through a dehydration series consisting of increasing concentrations of ethanol below. Proceed quickly as the NBT/BCIP stain is ethanol-soluble.

   30% EtOH, 3-5 s

   50% EtOH, 3-5 s

70% EtOH, 3-5 s
85% EtOH, 3-5 s
95% EtOH, 3-5 s
100% EtOH, 3-5 s (repeat once)

2. Immediately transfer slides to HistoClear II solution for 5 min. Repeat once. Stained sections are stable in HistoClear II solution.
3. Remove slides one at a time from HistoClear II and drain briefly on lint-free tissue.
4. Apply 2-3 drops of Permount mounting medium onto the surface of the slide.
5. Carefully lower a coverslip over the slide, lowering the cover slip gradually to ensure that air bubbles are not trapped. If bubbles are present, dip the mounted slide in HistoClear II, carefully slide off the coverslip, add the slide back to HistoClear II (Step J2) and repeat Step J3.
6. Allow slides to air dry for several h to overnight before imaging.

Data analysis

Figure 5. RNA *in situ* results on maize tissues. A. *DROOPING LEAF1* (*DRL1*; Strable *et al.*, 2017) in vegetative shoot apex showing hybridization in young leaves. B. *PHABULOSA* (*PHB*; Johnston *et al.*, 2014) in vegetative shoot apex showing hybridization in vasculature. C. *KNOTTED1* (*KN1*; Jackson *et al.*, 1994) in inflorescence showing hybridization in spikelet meristems. D. *KN1* in florets showing hybridization in floral meristems. Scale bars, 200 µm.

Recipes

1. FAA fixative
   50% Ethanol
   3.7% Formaldehyde
   5% Glacial Acetic Acid
2. 1× PBS buffer
   130 mM NaCl
   7 mM Na_2HPO_4
   3 mM NaH_2PO_4; pH 7.0
   Diluted from 10× stock solution

3. 2× carbonate buffer
   80 mM $NaHCO_3$
   120 mM Na_2CO_3
4. 10% Glacial Acetic Acid
5. 3 M NaOAc; pH 5.2
6. 4 M NH_4OAc
7. 1× TBS buffer
   100 mM Tris-HCl; pH 7.5
   150 mM NaCl
   Diluted from 10× stock solution
8. 1× TN buffer
   100 mM Tris-HCl; pH 9.5
   100 mM NaCl
9. Protease buffer
   100 mM Tris-HCl; pH 8.0
   50 mM EDTA; pH 8.0
10. 1× *in situ* hybridization salts
    3 M NaCl
    100 mM Tris-HCl; pH 8.0
    100 mM Na Phosphate; pH 6.8
    50 mM EDTA; pH 8.0
    Diluted from 10× stock solution
11. 0.2× SSC
    Diluted from 20× stock solution
12. 1× NTE buffer
    0.5 mM NaCl
    10 mM Tris-HCl; pH 7.5
    1 mM EDTA; pH 8.0
    Diluted from 5× stock solution

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Competing interests

We declare that we have no conflicting or competing interests with respect to the implementation of the protocol outlined herein.

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