RNA In Situ-Hybridization of Brachypodium whole mount leaf developmental zone

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protocol based on Huang et al. 2023 (https://doi.org/10.1186/s13007-023-01108-9)

adapted for microwave permeabilization from Chelysheva et al. 2024 (https://onlinelibrary.wiley.com/doi/10.1111/tpj.16792)

Published in Spiegelhalder et al. 2024 (https://journals.biologists.com/dev/article/doi/10.1242/dev.203011/361715/Dual-role-of-BdMUTE-during-stomatal-development-in)

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Notes

- Make sure to limit possible RNase contamination in your experiment: Clean your workspace with RNase Away; use filter tips for all steps; avoid touching the tubes/plates without gloves on
- Throughout the steps for this protocol I will write “tube” but it could also refer to a well from a 48 well plate
- When using a 48 well plate instead of tubes for the experiment, incubation on a tube rotator could not be done so the Eppendorf thermomixer with plate block set to 1000 rpm at RT (21°C) was used
- When using 2 ml tubes, a Fisher Scientific tube rotator set to speed 40 was used at RT
- All 37°C incubation steps were done with an Eppendorf thermomixer either with the tube or plate block
- Vacuum infiltration was done using a desiccator with a vacuum pump, plates or tubes were put in with the lid open

Materials

Equipment

- [ ] Tweezers
- [ ] Scalpel
- [ ] Small petri dish
- [ ] 48 well plate [optional]
- [ ] 2 ml Eppendorf tubes
- [ ] Desiccator with vacuum pump
- [ ] Eppendorf Thermomixer
- [ ] Tube rotator (or plate block for the Thermomixer)
- [ ] Pipettes and filter tips (Volumes 2 µl to 1 ml)
- [ ] Sterile Falcon tubes to prepare buffers

Day 1

- [ ] **Ethanol (100% needed for dilutions)**
  - [ ] 70% with milliQ water [can be prepared beforehand and stored at RT]
  - [ ] 50% with milliQ water [can be prepared beforehand and stored at RT]
  - [ ] 30% with milliQ water [can be prepared beforehand and stored at RT]
  - [ ] 10% with milliQ water [can be prepared beforehand and stored at RT]
- [ ] **Fixative FAA** [prepare fresh], 1 ml per tube or well

	final concentration	**required amount for 1 ml**
Formaldehyde solution (Sigma, F8775-25ML)	4% (v/v)	40 µl
Glacial acetic acid (Fisher Scientific)	5% (v/v)	50 µl
Ethanol	50% (v/v)	500 µl
Nuclease-Free Water (Thermo Fisher Scientific, AM9937)		410 µl

Day 2

- [ ] **Proteinase K** [can be prepared beforehand and frozen away in 1 ml aliquots at -20°C], 1 ml per tube or well

	final concentration	**required amount for 1 ml**
1M Tris-HCl, pH 8	0.1 M	100 µl
0.5M EDTA, pH 8	0.05 M	100 µl
Nuclease-Free Water		796 µl
Proteinase K (Thermo Scientific, EO0491)	80 µg/ml	4 µl

- [ ] **1X DPBS-T** [prepare fresh], 7 ml per tube or well

	final concentration	**required amount for 7 ml**
Tween20 (Sigma, 93773-250G)	0.1% (v/v)	7 µl
1X DPBS (Gibco, 14040-117)		6.993 ml

☐ **50% Ethanol / 50% DPBS-T** [prepare fresh], 1 ml per tube or well

	final concentration	**required amount for 1 ml**
1X DPBS-T	50% (v/v)	500 µl
100% Ethanol	50% (v/v)	500 µl

☐ **Fixative II** [prepare fresh], 1 ml per tube or well

	final concentration	**required amount for 1 ml**
Formaldehyde solution	4% (v/v)	40 µl
1X DPBS-T		960 µl

Day 3 / 4

☐ **5X SSC-T** [prepare fresh on Day 3, then store the rest at 4°C for Day 4], 12 ml per tube or well

	final concentration	**required amount for 12 ml**
20X SSC buffer (Invitrogen, AM9770)	25% (v/v)	3 ml
Tween20	0.1% (v/v)	12 µl
Nuclease-Free Water		8.988 ml

☐ **HCR Probe Hybridization Buffer** (Molecular Instruments)
☐ **HCR Probe Wash Buffer** (Molecular Instruments)
☐ **HCR Probe Amplification Buffer** (Molecular Instruments)
☐ For staining of cell walls **[optional]**
- ☐ **Calcofluor White** (Fluorescence Brightener 28, Sigma F3543-1G)
- ☐ **SCRI Renaissance 2200** (Renaissance Chemicals)

Day 1 - Fixation, Dehydration and Permeabilization

1. Freshly prepare Fixative FAA and put it into a 2 ml tube you will use for the sample
2. Clean tweezers and a fine scalpel with RNase Away
3. Carefully pull out a young, developing leaf from the plant with the tweezers and cut off the bottom 3-5 mm (developmental zone) from the leaf, put it in a fresh petri dish and immediately put a few drops of the Fixative FAA on it
4. Cut the developmental zone once longitudinally and then once vertically and transfer the pieces into the tube containing the **Fixative** FAA
5. In the fume hood, apply and release vacuum several times until the leaf pieces don't float up anymore
6. Leave the samples in Fixative FAA for at least an hour at RT (I usually do 3h). Can also be stored in Fixative FAA overnight at 4°C
7. Dehydrate and **permeabilize** the sample in a series of steps using different ethanol concentrations. For each concentration, microwave the sample 5x at 180W for 30s and flick the tube softly after each of the 30s
    a. 10% Ethanol
    b. 30% Ethanol
    c. 50% Ethanol
    d. 70% Ethanol
8. Store the samples in 70% Ethanol at -20°C overnight (or up to several weeks)

Day 2 - Rehydration, Digestion, Fixation, (Pre)-Hybridization

9. Remove samples from -20°C and allow them to reach RT
10. **Rehydrate** the samples through a series of washes at RT on the tube rotator
    a. 50% Ethanol / 50% DPBS-T for 15 min
    b. 100% DPBS-T 2x for 15 min. During the first incubation step thaw an aliquot of 1 ml Proteinase K by taking it out from -20°C
11. Remove DPBS-T and incubate with Proteinase K at RT by applying vacuum for 5 minutes. In the meantime, heat up the thermomixer to 37°C. Release the vacuum and **digest** for 25 min at 37°C on the thermomixer. Agitate the samples every 5-10 min
12. Wash 2x for 15 min in DPBS-T at RT on the tube rotator
13. Incubate with Fixative II at RT by applying vacuum for 10 minutes in the fume hood. Release the vacuum and **fix** the tissue for 20 min at RT on the tube rotator. Meanwhile, prepare two 500µl aliquots of HCR Probe Hybridization Buffer: Leave one at RT for step 15 and put the other at 37°C for step 16
14. Wash 2x for 15 min each in DPBS-T at RT on the tube rotator
15. Remove DPBS-T and replace it with 500µl of HCR Probe Hybridization Buffer (RT). Apply vacuum in the fume hood for 10 minutes. Release the vacuum and **pre-hybridize** by incubating for 1h at 37°C in the thermomixer with shaking (1000 rpm)
16. Prepare probe solution(s) by adding 0.8 pmol of each probe set (i.e. 2 µl of the 1 µM stock) to 500 µl of HCR Probe Hybridization Buffer at 37°C
17. Remove pre-hybridization solution as much as you can. Be careful to not take up the leaf pieces while you pipet. Add the probe solution and hybridize by incubating overnight (~22h) at 37°C in a thermomixer with shaking (1000 rpm)

18. Store an aliquot of HCR Probe Wash Buffer (2 ml per sample tube) at 37°C overnight (or at least a few hours before) to pre-heat

Day 3 - (Pre)-Amplification (start about 22h after Day 2)

19. Aliquot HCR Amplification Buffer (per sample tube 1 aliquot with 500 µl and another with 250 µl) and leave out to warm up to RT
20. Remove excess probes by washing 4x for 15 min each with 500 µl of HCR Probe Wash Buffer (stored overnight at 37°C incubator) at 37°C in a thermomixer with shaking (1000 rpm)
21. Wash samples 2x for 5 min each with 1 ml of SSC-T buffer at RT in a thermomixer with shaking (1000 rpm). Store the rest of the SSC-T buffer at 4°C for Day 4
22. Take out hairpins h1 and h2 and let them thaw (keep cool once thawed)
23. Replace SSC-T with 500 µl of HCR amplification Buffer (aliquoted in step 22). Apply vacuum for 10 minutes. Release vacuum and **pre-amplify** on tube rotator at RT for 50 min
24. While samples pre-amplify, prepare 6 pmol of hairpin 1 and 6 pmol of hairpin 2 (i. e. 5 µl of the 3 µM stocks from Molecular Instruments) in separate tubes. Snap cool the hairpins by heating at 95°C for 90 seconds then keep them in a dark drawer at RT for 30 min
25. Prepare the amplification solution by combining snap cooled hairpin 1 and hairpin 2 in 250 µl of HCR Amplification Buffer (aliquoted in step 22) at RT
26. Remove the pre-amplification solution and Amplify by incubating in hairpin solution for two days (>40h) in the dark at RT

Day 5 - Imaging (start about 42h after end of Day 3)

27. Take out SSC-T buffer from 4°C and let it warm up to RT
28. Remove excess hairpins by washing with 1 ml of SSC-T buffer at RT in a thermomixer with shaking
    a. 2x for 5 min each
    b. 2x for 30 min each
    c. 1x for 5 min
29. [optional] If necessary, use cell wall staining to make cell outlines visible on the confocal microscope. I used Calcofluor White (Fluorescence Brightener 28) or SCRI Renaissance 2200 staining (both stain beta 1,4 or 1,3 linkages → cellulose)
30. **[optional] Calcofluor White cell wall stain**
    a. Prepare 0.01% Calcofluor White in SSC-T buffer (10 mg in 100 ml, can be stored at 4°C for weeks, keep dark)
    b. 1 min incubation of leaf pieces in 0.0001% Calcofluor (10µl of 0.01% stock + 990µl SSC-T buffer)
    c. wash 3x with 1 ml SSC-T buffer

31. **[optional] SCRI Renaissance 2200 cell wall stain**
    a. Prepare 0.001% (v/v) SCRI Renaissance 2200 in SSC-T buffer (can be stored at RT in a dark drawer)
    b. 1 min incubation of leaf pieces in 0.001% SR 2200 (100 µl)
    c. wash 3x with 1 ml SSC-T buffer
32. **Imaging** on a confocal microscope
    a. Clean scissors with RNaseAway, cut off the tip of a 1ml pipette tip and use this to transfer the sample with some SSC-T buffer on a microscopy slide
    b. We currently use the amplifier 488 as B1 (green) so to detect the probe signal, use settings for Alexa488 fluorophore
    c. Amplifier 594 as B2 (red) → Alexa594 settings on microscope
    d. Calcofluor White and SCRI Renaissance 2200 work with Alexa405 settings, I recommend to minimize the emission range for this one since the dyes are quite strong