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Immunohistochemistry of plant gall tissue

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We use this protocol and it's working

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Abstract

Here we describe a method to fix, antibody stain, clear, and mount plant gall tissue for light microscopy. The protocol was developed to visualize effector protein distribution within galls induced by the aphid *Hormaphis cornu* on leaves of *Hamamelis virginiana* (Witch Hazel).
The protocol employs a partial cell wall digestion step to allow antibody penetration into 100 µm tissue sections. We also employed secondary antibodies tagged with DNA hairpin probes, which allows signal amplification with the Hybridization Chain Reaction, to visualize low-abundance proteins. Solvent-based dehydration, clearing, and mounting substantially reduced plant tissue pigmentation and autofluorescence.

Guidelines

- All steps are performed at room temperature on a rotary shaker set to 25 RPM, unless otherwise indicated.

- Each well of the PYREX 9 Depression Glass Spot Plate can comfortably hold 200 µl of solution without spillage. We use 200 µl volumes for wash steps and 100 µl for antibody staining. We do not recommend placing more than a single tissue section per well.
To minimize evaporation from sample wells, we recommend; 1. Cover each well with a coverslip held in place with Blu Tack and, 2. A minimal volume of 100 µl per well.

- To minimize damage to agarose-embedded tissue, we recommend using pipettes with disposable tips rather than disposable transfer pipettes to add/recover solutions to/from the sample wells .

- Ethanol, Xylene and DPX are flammable and can be toxic if inhaled. Please use full PPE including a mask, lab coat and gloves while handling.

-If using secondary antibodies with fluorescent tags, incubate samples in the dark starting at Step #21.

Materials

1. Pipettors (1 mL, 200 µL, 20 µL, and 10 µL)
2. Disposable tips (1 mL, 200 µL, 20 µL)
3. 32% paraformaldehyde (Electron Microscopy Services, catalog #15714)
4. 8% glutaraldehyde (Electron Microscopy Services, catalog #16019)
5. 10% Triton X-100 (Sigma-Aldrich, catalog # X100)
6. 10X Phosphate Buffered Saline (Corning, catalog # 46-013-CM)
7. Nutator
8. Agarose (Fisher Scientific, catalog # BP160-100)
9. PYREX Round Media Storage Bottles, with GL45 Screw Cap (Corning, catalog # 1395-100)
10. Microwave
11. 35 × 10 mm petri dish (Corning Falcon, catalog # 353001)
12. Feather Single-Use Scalpel # 10 (Fisher Scientific, catalog # 08-927-5A)
13. Fine paintbrush
14. Forceps
15. Dissecting microscope
16. Superglue
17. Leica VT1200S Vibratome
18. PYREX 9 Depression Glass Spot Plate (Corning, catalog # MC-9DGSP)
19. Cellulase (Onozuka R 10, Fisher Scientific, catalog # 50-213-229)
20. Macerozyme (R 10, Fisher Scientific, catalog # 50-488-794)
21. 2-(N-Morpholino)ethanesulfonic acid (MES; Sigma-Aldrich, catalog # M3671)
22. Shaking incubator set to 37°C
23. Hybridization Chain Reaction (HCR) antibody buffer (Molecular Instruments)
24. HCR amplification buffer (Molecular Instruments)
25. Heatblock set to 95°C
26. Primary antibody to an epitope of interest
27. Secondary antibody that binds the primary antibody
28. Shaking incubator at 4°C
29. Blu Tack Reusable Adhesive or something similar
30. Glass coverslips (Fisherbrand No. 1 and No. 1.5)
31. Sodium chloride sodium citrate solution (Promega, catalog # V4261)
32. Tween 20
33. Hoechst 33342 (Life Technologies, catalog #H3570)
34. Ethanol
35. Xylene
36. DPX Slide Mounting Medium, Histological Grade, Liquid (Sigma-Aldrich, catalog # 06522)
37. Amber bottle with glass dropper to hold DPX (VWR catalog # 16199-037)
38. Kimwipes or similar lab wipes

Fixation

3h

1 Prepare fixative solution: 1 X PBS with 2% paraformaldehyde, 0.5% glutaraldehyde, and 0.1% Triton X-100. Prepare sufficient volume to submerge plant tissue. Use within 1 day of preparation.

2 Trim plant material or use whole leaves with incipient galls. Place tissue into 2 ml round bottom Eppendorf tubes for samples smaller than 5 mm or 50 ml conical tubes for larger samples. Add prepared fixative to fully submerge the tissue and incubate at room temperature for 2 hours on a nutator.

3 Discard fixative and wash fixed samples in 1 X PBS with 0.1% Triton X-100 (PBT) for 20 minutes. Use 2ml PBT for smaller samples and 10 ml PBT for samples in conical tubes.

2h

4 Repeat step #3 twice for a total of 3 washes.

1h

5 Proceed immediately to tissue embedding or store samples in PBT at 4°C. Do not embed if you do not plan to prepare slices for antibody staining within ~24 hours because samples embedded in agarose are easily contaminated.
For long term storage at 4°C, refresh the PBT every 2-3 days. We have stored samples in PBT at 4°C for several months without loss in imaging quality.

Agarose embedding and sectioning

2h 10m

6 Measure agarose powder to make an 8% solution. Add agarose to a Pyrex Round Media Storage Bottle and then add the correct volume of 1X PBS. Melt agarose in a microwave set to low power (40-60%) for ~7 minutes with intermittent mixing to evenly dissolve the agarose. Avoid introducing bubbles during mixing. Make sufficient agarose solution for each sample to receive 5 ml plus 50% excess (7.5 ml per sample).

7 Allow agarose solution to cool slightly. The agarose is ready to pour when you can hold the bottle comfortably against your inner wrist for 5 seconds. Place fixed tissue in a 35 × 10 mm petri dish and slowly pour cooled agarose solution over the sample. Use a fine paintbrush or forceps to orient the sample as desired. Ensure that the tissue is embedded within 5 mm of agarose to allow ample space to mount and prepare an agarose block as in step 9 below. Work quickly while the agarose is still molten.

10m

8 Cool samples rapidly on ice. Then place at 4°C for at least 2 hours to allow agarose gellation.

2h

9 Use a fresh scalpel to excise the area with the sample plus a 6-8 mm boundary. Place the trimmed agarose block into cold 1X PBS in a Petri dish and further trim into a parallelogram shape with a wide base for easy mounting. Keep the sample close to the top of the trimmed parallelogram.
Trimming steps are best performed with the aid of a dissecting microscope. Keep trimmed blocks submerged in cold PBS and proceed immediately to sectioning.

10 Set the vibratome to make sections of 100 µm thickness at a cutting speed of 0.8-0.9 m/sec. Higher speeds tend to dislodge the block from the mounting plate during sectioning. Slower speeds are fine for sectioning but add time with no apparent benefit to slice quality. In our experience, agarose slices thinner than 50 µm are too fragile to handle in downstream steps.

11 Glue the trimmed block onto a vibratome mounting plate with minimal superglue. The glue should remain underneath the agarose block and not coat the sides.
Allow glue to set for 2 minutes at room temperature.

12 Place the mounting plate with glued sample into the cutting chamber on the vibratome. Cover the mounting plate and block with cold 1 X PBS. Set the cut start and end points and blade height. Collect tissue sections with a fine paintbrush directly into cold 1 X PBS dispensed into a multi-well dish. (Agarose sections may be stored for up to 1 day at 4°C prior to cell wall digestion. We have not tested longer storage periods.)

Partial cell wall digestion

2h 30m

13 Prepare the cell wall digestion solution: 0.2% cellulase + 0.15% macerozyme in 2 mM MES, pH 5.0. Keep the enzyme solution on ice and use within 1 day.

14 Transfer sections to individual wells of a PYREX 9 Depression Glass Spot Plate. Wash 2x, 20 minutes each with 200 µl 2 mM MES, pH 5.0. 40m

15 Add 500 µl cell wall digest solution to each section, transfer dish to a 37°C platform incubator set to rotate at a speed of 25 RPM for 30 minutes. 30m

16 Remove the cell wall digest solution and wash sample with 200 µl 2 mM MES. Remove this wash and then perform 2x, 20 minute washes with 200 µl 2 mM MES. 40m

17 Equilibrate samples for antibody staining with 2X 20 min washes in 200 µl of 1X PBS. Proceed immediately to antibody staining. 40m

Antibody staining (modified from the "on slide" protocol provided by Molecular Instruments)

4d 18h 51m 30s

18 Block samples for 1 hour in 100 µl HCR antibody buffer. Remove the buffer. 1h

19 Add primary antibodies diluted in HCR antibody buffer to the appropriate concentration. Incubate overnight at 4°C on a rotary platform set to 25 RPM. To prevent sample loss by evaporation, cover each well with a coverslip held in place with Blu Tack (Bostick). We use 100 µl of diluted antibody solution per section. 16h

20 Remove primary antibody. Perform four washes for 20 minutes each with 200 µl of PBS with 0.1% Tween 20 (PBST). 1h 20m

21 Add 100 µl of secondary antibodies diluted in HCR antibody buffer to the appropriate concentration and incubate for 4 hours at room temperature (or overnight at 4°C) on a rotary platform. 4h

22 Remove secondary antibody and perform a single 20 minute wash with 200 µl of PBST followed by 10 minute 200 µl washes in a series (0.5x, 1x, 2.5x, 5x) of sodium chloride sodium citrate solution with 0.1 % Tween 20 (SSCT). 1h

22.1 Bring HCR amplification buffer to room temperature from 4°C during the previous step. You will need a total of 450 µl HCR amplification buffer per section. Prepare 10% excess buffer. 30m

23 After the final buffer exchange, discard the 5x SSCT, add 200 µl of the HCR amplification buffer prepared in step #22.1 and incubate at room temperature for 30 minutes. 30m

24 Prepare HCR hairpin probes following the manufacturer's instructions as follows: heat at 95°C for 90 seconds and then cool to room temperature in the dark, about 30 minutes. Add cooled hairpin probes to 250 µl of the room temperature HCR amplification buffer, mix 10 times by pipetting with a P200 pipette and add to the samples. 1m 30s

25 Cover samples with a coverslip held in place with Blu Tack and incubate at room temperature for 16 hours, without agitation, in the dark. 16h

26 Remove amplification solution and perform two washes in 200 µl 5x SSCT for 30 minutes followed by washes for 10 minutes each in an SSCT series (2.5x, 1x, 0.5x). Use 200 µl for each SSCT wash.

27 Optional: stain samples with 100 µl of 1ug/mL Hoechst 33342 in 0.5X SSCT for 20 minutes and then wash 2x with 200 µl 0.5X SSCT for 20 minutes. 1h

28 To clear and mount samples for imaging, incubate samples for 10 minutes each in an ethanol dehydration series (30%, 50%, 75%, 95%, 100%). Use 200 µl of each ethanol wash. Replace the final 100% ethanol with 200 µl xylene for 10 minutes. 1h 30m

28.1 Repeat the 200 µl xylene wash once for a total of two incubations.

29 Using the guidelines below, mount dehydrated samples on a glass slide in a sufficient volume of DPX to just cover the sample underneath a glass coverslip. We recommend storing DPX in a glass bottle equipped with a glass dropper and bulb.

29.1 With a pair of forceps, place the dehydrated sample in the centre of the slide.

29.2 Before dispensing DPX, clear the dropper of any bubbles as follows: Squeeze the dropper to expel any air, then slowly allow the DPX to fill the dropper halfway taking care not to fill the bulb with the solution. While holding the bulb, slowly withdraw the dropper from the bottle and dispense ~2 drops on top of the dehydrated sample on the slide.

29.3 Place a clean glass coverslip over the sample taking care to avoid introducing any bubbles. Wait 2 seconds and gently tap the coverslip once with the back end of a pair of forceps to evenly distribute the DPX underneath the coverslip. There should be no air gaps on the edges of the coverslip.

29.4 Wipe off excess DPX with a Kimwipe and set the slide on a rack to cure. We use empty pipet tip refill trays as racks for this step.

30 Cure the slide in the dark for at least 72 hours in a chemical fume hood prior to imaging. 3d

30.1 Any excess DPX remaining on the slide can be cleaned with a Q-tip dipped in xylene.

Protocol references

Molecular Instruments Generic on slide Immunofluorescence protocol

https://files.molecularinstruments.com/MI-Protocol-2IF-GenericSlide-Rev3.pdf