Chapter 8

Immunolocalization in Secondary Xylem of *Populus*

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Abstract

Immunolocalization can be used to precisely visualize the location of specific proteins, cell wall components, or any other molecules within cells or tissues for which specific antibodies are available. Here we describe an immunolocalization protocol for tissue sections of woody *Populus* stems. The protocol includes descriptions of the required sectioning, fixation, probing, detection, and imaging parameters, as well suggested controls useful in interpreting results.

Key words Antibodies, Antigen, Cell wall, Forest trees, Immunolocalization, Wood development

1 Introduction

Immunolocalization has been a primary tool in animal and plant research. There are numerous variations on the technique, but in general immunolocalization makes use of antibodies to detect and image the location of specific molecules within in a tissue (Fig. 1). In plants, a wide range of antibodies have been raised against individual moieties contained within proteins, carbohydrates, chromatin marks, and even small molecules including hormones. To probe and detect these epitopes in plant tissues, typically the tissue is fixed, embedded and sectioned. The sections are affixed to slides and probed with the antibody (termed the primary antibody). Alternatively, sections or small pieces of tissue can be probed as "whole mounts" without being affixed to slides, as in the protocol here. Two different methods can be used to then visualize where the primary antibody has localized the antigen. In colorimetric methods, a secondary antibody recognizing the primary antibody is conjugated to an enzyme (e.g., alkaline phosphatase) and used to probe the section. A substrate is then provided which, when acted upon by the conjugated enzyme, produces a colored reaction product that can be visualized under the microscope. An alternative method is to use a fluorescent reporter for visualization. In this approach, a fluorophore is conjugated to the secondary antibody,

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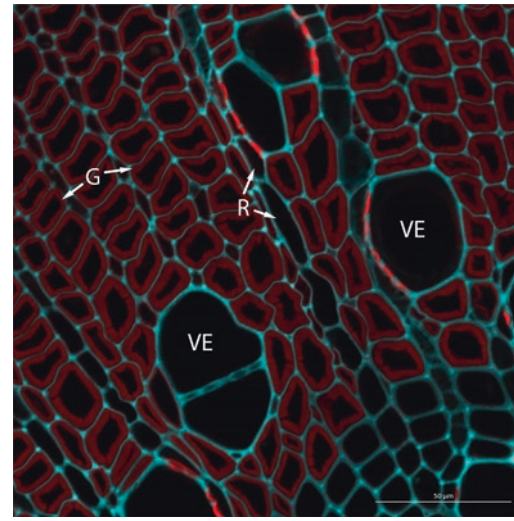

Fig. 1 Confocal image of immunolocalization in secondary xylem of *Populus*. Vibratome sections were prepared from *Populus* stems of a genotype overexpressing the ARBORKNOX2 transcription factor, and subjected to the immunolocalization protocol described here. The primary antibody used was JIM14 at 1:500 dilution (Complex Carbohydrate Research Center, University of Georgia, Athens, GA), whose recognition includes arabinogalactan protein epitopes [1]. Secondary antibody was biotin-xx-goat anti-rat IgG at 1:400 dilution (Life Technologies A10517). Detection was with 4 μl/ml of Streptavidin Alexa Flour 488 (Life Technologies S11223). *Red signal* is from Alexa Flour 488, which includes G-layers of tension wood fibers. *Blue signal* is autofluorescence resulting from UV excitation. Imaging was performed using a Zeiss LSM 710 confocal microscope. *G* g-layer, *R* ray, *VE* vessel element

which can be visualized using a confocal or epifluorescence microscope. A modification of this approach is to conjugate biotin to the secondary antibody, and perform detection using a fluorophore conjugated to streptavidin, which has high affinity to biotin. This method essentially serves to amplify the observed signal, and is utilized in the protocol here (Fig. 1).

While relatively simple in concept, immunolocalization must be optimized for different species, tissues, antibodies and epitopes. Xylem tissues from woody plants are challenging subjects. When sectioned, xylem has a tendency to curl and it can be difficult to firmly adhere sections to slides. Additionally xylem contains a number of secondary compounds and has cells with complex, lignified cell walls. These features frequently cause problems including nonspecific binding of antibodies, high background autofluorescence in fluorescence-based detection and difficulties in reaching the target epitope. Thus, the protocol here has been optimized to reduce nonspecific binding in xylem using complex blocking reagents and copious washing steps. Additionally, the

protocol makes use of a fluorophore for detection that has excitation and emission spectra that have acceptable levels of autofluorescence in xylem. Finally, a whole mount procedure is used to avoid problems with adherence of sections to slides, and the sectioning and fixation procedures optimize perfusion of paraformaldehyde through the complex woody cell wall thus enhancing antibody access to the target epitope.

The immunolocalization protocol here makes use of whole-mount sections of stem tissues from *Populus* seedlings, and has been used previously to visualize both specific protein and cell wall epitopes [2]. The same protocol could likely be extended to xylem of other woody species.

2 Materials

Having a high quality primary antibody is critical for success. If working with an untested antibody, it is recommended that specificity be tested using Western blots. Note that formaldehyde is a carcinogen and should be handled using appropriate personal protective equipment within a fume hood. Formaldehyde must be disposed of as hazardous waste following disposal regulations.

2.1 Sectioning and Fixation

1. Freshly prepared FAA fixative on ice: 11.25 ml EtOH, 10 ml dH_2O, 1.25 ml acetic acid, 2.5 ml formalin (BP531 Fisher).
2. PBT buffer: 5 ml PBS, 45 ml dH_2O, 100 µl Tween 20.
3. 1× blocking buffer: 2.5 ml 10× PBS, 5 ml 10% Fish Gelatin, 2.5 ml 10% BSA, 15 ml dH_2O, 50 µl Tween 20.
4. Required equipment: Vibratome, double edged razor blades, fine tipped paintbrushes, deionized water, cyanoacrylate adhesive (e.g., Crazy Glue™), lab quake or similar mixer, 5 ml round bottom tubes, 1.5 ml snap top tubes.

2.2 Probing, Detection and Imaging

1. PBT buffer: 5 ml PBS, 45 ml dH_2O, 100 µl Tween 20.
2. 1× blocking buffer: 2.5 ml 10× PBS, 5 ml 10% Fish Gelatin, 2.5 ml 10% BSA, 15 ml dH_2O, 50 µl Tween 20.
3. Primary antibody.
4. Secondary antibody that recognizes primary antibody, conjugated to biotin.
5. Strepavidin conjugated Alexa 488 (ThermoFisher S11223).
6. Required equipment: Labquake or similar mixer, microscope slides and coverslips, epiflourescence or confocal microscope.

3 Methods

3.1 Sectioning and Fixation

1. Place the vibratome directly adjacent to a fume hood. Add water to the vibratome and attach the blade as per manufacturer instructions. The water level should cover the cutting edge of the razor blade. Note that the blade angle, speed of blade advance, and amplitude of blade can all be adjusted to optimize sectioning.
2. Prepare FAA fixative and place it in 2–3 ml round bottom 5 ml tubes on ice inside the fume hood.
3. From a healthy tissue culture grown poplar seedling (or similar plant), use a razor blade to cut a segment from the stem to be sectioned. The segment should be no more than ~6 mm long. For difficult to section woody stems (e.g., soil grown poplar seedlings, or those over 5 mm in diameter), cut longitudinally into two half-rounds (*see* **Note 1**).
4. Affix the stem segment to the sectioning block by placing a drop of cyanoacrylate adhesive (e.g., super glue) on the block by dipping the base of the stem segment in the glue, and then positioning the segment vertically near one edge of the block. Avoid placing the stem segment in a drop of glue that has been placed on that block as this can cause ineffecient glue drying. Allow the glue to dry for ~2 min before placing the sectioning block into the chuck of the vibratome.
5. Adjust the sample height so that the blade is just above the height of the sample.
6. Use the control switches on the vibratome to control the blade movement. Up will fast forward, down will reverse, and up/release will advanse the blade slowly to cut.
7. Press up on the control switch to move the blade towards the sample, then use slow advance as the blade cuts the section. Before reversing the blade, adjust the sample height down by 20–30 μm to avoid scraping the sample as the blade returns to the starting position.
8. Raise the block by 50–100 μm and take a fresh cut to leave a smooth surface.
9. After making the first full section, use a small paintbrush to retrieve it from the water bath and transfer it to fixative on ice.
10. The blade must be sharp, and should be changed every few sections or every section for woodier tissue.
11. Once you have collected as many sections as desired, remove the round bottom tube from ice and place it into a vacuum chamber at room temperature in the fume hood. Draw a vacuum and incubate for 45 min (*see* **Note 2**).

12. Repeat until all tissue types have been collected. Note, if there are two halves of the same stem that both need to be cut, they can both be placed onto the cutting block and sectioned at the same time. The blade dulls very easily and cutting half rounds helps to prevent that. Using a fresh blade for every cut helps. If the sample bends as it is cut, a convex or incomplete section results and several passes of the remaining tissue are sometimes required before another full section can be obtained. Smaller samples can be embedded in agarose before being affixed to the block for sectioning.

3.2 Blocking and Probing

1. After fixing for 45 min, remove the samples from the vacuum and place them on ice. Step the samples slowly into PBT as follows. Over the course of ~5 min, add PBT slowly by drops to equal the volume of fixative. Remove 1 ml of the solution and repeat slowly adding drops of PBT for a total of 1 ml. This process is repeated several times until the schlieren lines are no longer apparent.
2. Remove the remaining fixative/PBT solution and replace it with 2 ml of PBT. Cap the tube and incubate it with gentle mixing 15 min at room temperature.
3. Discard the PBT wash solution, add 2 ml of PBT and incubate at room temperature with gentle mixing for 15 min. Repeat for a total of 4, 15 min washes. Distribute the sections into Eppendorf tubes. (Note: Include at minimum one treatment for a no-primary antibody control, and one or more treatments for the primary antibody. If the primary antibody has not been previously optimized in your system, consider testing a range of primary antibody concentrations.)
4. Remove the PBT and replace it with a small volume of blocking buffer to rinse. Replace with 1 ml of blocking buffer and incubate at room temperature for ≥1 h.

3.3 Primary Antibody Incubation

1. Primary antibodies are typically shipped and stored at high concentration and need to be diluted to effective working concentrations by the user. A good rule of thumb is to start out with a manufacturer's recommended final working concentration and then try one sample at tenfold higher concentration, and one sample at tenfold lower concentration if you have enough antibody on hand (*see* **Notes 3–5**). Briefly centrifuge the primary antibody in a microcentrifuge before using to avoid pipetting any aggregates.
2. Place the Eppendorf tubes with sample and blocking buffer on ice. Pipette the required volume of primary antibody into each tube, with the exception of the no-primary antibody control (*see* below). Place the samples on a lab quake or similar rocker and mix gently at 4° overnight.

3. The next day, remove the tubes from the 4° chamber. Remove the block/antibody solution and perform one PBT wash. Then perform a series of four PBT washes of 15 min each, at room temperature with gentle mixing.
4. Remove the PBT and replace it with a small volume of blocking buffer to rinse. Replace with 1 ml of blocking buffer and incubate at room temperature for ≥1 h.

3.4 Secondary Antibody Incubation

1. The secondary antibody should recognize antibodies from the species used to produce the primary antibody. For example, if the primary antibody was produced in rabbits, the secondary antibody should recognize rabbit IGG (sometimes termed an anti-rabbit secondary antibody).
2. Secondary antibodies are typically purchased in a highly concentrated form and need to be diluted to effective working concentrations. Follow the manufacturers recomendations or test a dilution series of concentrations.
3. The secondary antibody used in this protocol should be labeled with biotin.
4. Briefly spin the secondary antibody in a microcentrifuge, and pipette the desired volume into your tube containing sample and blocking buffer, on ice.
5. Incubate overnight at 4° C with gentle mixing.

3.5 Dectection and Imaging

1. Rinse your samples one time with PBT, then perform four PBT washes, 15 min each at room temperature with gentle mixing.
2. Rinse one time with a small volume of blocking buffer, then replace with 1 ml of fresh blocking buffer and incubate at room temperature with gentle mixing for >30 min.
3. Add streptavidin-fluorophore conjugate to desired concentrations. In our lab, we use 4 μl/ml streptavidin-Alexa 488. Wrap each tube in foil to protect from direct light, to avoid photobleaching of the fluorophore.
4. Incubate at room temperature for ≥30 min with gentle mixing.
5. Rinse one time with 1 ml PBT.
6. Wash four times with 1 ml PBT, 15 min each at room temperature with gentle mixing.
7. Sections are now ready to be imaged by confocal or epifluorescence microscopy.

4 Notes

1. A potential area requiring optimization is the sectioning of xylem tissue. The best settings for vibratome sectioning must be experimentally determined for specific samples and tissues. In general, the vibratome is limited by the thickness and density of what it can section, so if a sample is too large or hard to section, partial dissection may be beneficial (e.g., instead of sectioning the entire circumference of a stem, bisect it longitudinally and section half the stem). Alternatively, samples that are not rigid enough can be embedded in agarose before being sectioned.
2. Fixation can be optimized, although in this case the whole mount sections are relatively uniform and cells are relatively accessible to fixative. If the reader is working with a readily transformed species and needs to determine protein localization, an alternative approach is to visualize recombinant, fluorescently labeled proteins in whole mount sections [3]. Consulting other protocols for plant immunolocalization may be useful when adapting or optimizing your protocol for other sample types [4].
3. The most unpredictable part of immunolocalization using this protocol will be the primary antibody. General information about working with and characterizing antibodies is available [5]. If you are working with a new antibody, it is highly recommended that you test a wide range of concentrations, and include a well-characterized antibody as a positive control in your initial experiments.
4. Several on-line resources are available for finding antibodies of interest. For example, a wide range of antibodies recognizing cell wall epitopes is available through the Complex Carbohydrate Research Center at the University of Georgia (http://www.ccrc.uga.edu/~mao/wallmab/Antibodies/antib.htm). Examples of immunolocalization for both protein and cell wall epitopes can be seen in [2].
5. Additional experimental validation of a new antibody such as western blotting can also helpful in determining specificity and estimating titer. In all experiments, a "no-primary antibody" treatment should be included, to determine the extent and nature of background fluorescence from autofluorescence or nonspecific sticking of the secondary antibody or streptavidin-fluorophore conjugate. In general, washes can be extended in time or number without significant loss of signal.

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