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Title: Fluorescent Immunolocalization of Arabinogalactan Proteins and Pectins in the Cell Wall of Plant Tissues

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Author Questionnaire

1. **Microscopy**: Does your protocol involve video microscopy, such as filming a complex dissection or microinjection technique? **Yes**

   If **Yes**, can you record movies/images using your own microscope camera?

   Yes

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes**

   If **Yes**, we will need you to record using screen recording software to capture the steps. If you use a Mac, QuickTime X also has the ability to record the steps.

3. **Filming location:** Will the filming need to take place in multiple locations? **Yes**

   If **Yes**, how far apart are the locations? **All in same building**

Introduction

1. Introductory Interview Statements

Videographer: Interviewee headshots are required. Take a headshot for each interviewee.

Authors: While filming the interview portion, our videographer will also photograph you for the **JoVE Dedicated Author Webpage**. Please look at this **example**. For questions about the author profile pages and pictures, please contact **author.liaison@jove.com**.

Authors: Please memorize the interview statements prior to your filming day.

1.1. **Sílvia Coimbra:** The amount of information from only one experiment is tremendous. One can not only detect the presence of these cell wall polymers but know exactly how they are distributed determine their abundance **[1]**.

1.1.1. INTERVIEW: Named author says the statement above in an interview-style statement while looking slightly off-camera.

1.2. **Ana Marta Pereira:** This method is designed in such a way that it can be applied to almost any kind of plant tissue from any species one may want to analyze **[1]**.

1.2.1. INTERVIEW: Named author says the statement above in an interview-style statement while looking slightly off-camera.

1.3. **Mario Costa:** Immunolocalization on plant tissues involves a lot of meticulous work, difficult to explain without actually seeing what happens **[1]**.

1.3.1. INTERVIEW: Named author says the statement above in an interview-style statement while looking slightly off-camera.

Answers have been edited for length, clarity, and consistency with journal style guidelines.

Protocol

Please use this draft script to help you prepare for filming day.
- Filming should take no more than 10 minutes per step. If a step will take more than 10 minutes, prepare the product from that step before filming begins.

2. Sample Fixation and Dehydration

2.1. Before collecting the plant tissue samples, fill a glass vial with enough cold fixative solution to completely submerge all the samples **[1-TXT]**. Then, select the plant tissues to be analyzed, and trim the samples to a size of no more than 16 square millimeters **[2]**. Immediately immerse the samples in the fixative solution **[3]**.

2.1.1. Talent prepares cold fixative solution. **TEXT: Fixative solution:; 2% formaldehyde (w/v); 2.5% glutaraldehyde (w/v); 25 mM PIPES pH 7; 0.001% Tween-20 (v/v)** *Video editor, please keep this text onscreen for the next two shots.*

2.1.2. Talent trims plant tissue samples. *Videographer: This is one of the most important steps for viewers to see.*

2.1.3. Talent places plant tissue samples in glass vial of fixative.

2.2. **Ana Marta Pereira:** Glutaraldehyde and formaldehyde are both excellent fixatives. But both are hazardous and must be handled in the flow hood **[1]**.

2.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot. *Videographer: Since this is a warning statement, have the talent look more directly at the camera compared to other interview statements.*

2.3. Transfer the vial to a vacuum chamber, and slowly apply vacuum **[1]**. When the pressure reaches negative 60 kilopascals **[2]**, the floating material in the vial will start to sink to the bottom **[2b]**. Maintain the pressure in the chamber at no more than negative 80 kilopascals. The sinking process may take up to two hours **[3]**.

2.3.1. Talent transfers vial to vacuum chamber and slowly applies vacuum.

2.3.2. ECU: samples in vial sinking to the bottom.

2.3.2.B Added shot: samples in vial sinked after two hours.

2.3.3. Vacuum chamber showing vacuum pressure gauge in correct range.

2.4. After the samples have been in the vacuum chamber for 2 hours, slowly release the vacuum **[1]**. Seal the glass vial **[2]** and refrigerate it overnight at 4 degrees Celsius **[3]**.

2.4.1. Talent releases vacuum, and the gauge shows the pressure in the chamber slowly increasing.

2.4.2. Talent removes vial from chamber and seals it.

2.4.3. Added shot: Talent places vials in refrigerator.

2.5. After the overnight fixation, discard any samples that did not sink to the bottom of the vial **[1]**. Wash the remaining samples with 25 millimolar PBS for 10 minutes, and then wash them in 25 millimolar PIPES buffer for 20 minutes **[2]**.

2.5.1. Talent removes floating samples from vial and discards them.
2.5.2. Talent begins process of washing the remaining samples.

2.6. **Mario Costa:** It is extremely important to remove all floating samples, because its highly probable the fixative has not penetrated these sample **[1]**.

2.6.1. INTERVIEW: Named author says the statement above in an interview-style statement while looking slightly off-camera.

2.7. Dehydrate the samples in an ethanol series **[1-TXT]**, immersing the samples for 20 minutes **[2]** at each ethanol concentration **[3]**.

2.7.1. Labeled containers of ethanol prepared for dehydrating the samples. **TEXT: Ethanol series: 25%, 35%, 50%, 70%, 80%, 90%, 100% (3 times)** *Video editor, please keep this text onscreen for the next shot.*
2.7.2. Talent immerses plant tissue sample in 25% ethanol. *Videographer: This is one of the most important steps for viewers to see.*
2.7.3. Added shot: Talent immerses plant tissue sample in 100% ethanol. *Videographer: This is one of the most important steps for viewers to see.*

~~2.8. After dehydration, immediately transfer the samples to labeled glass vials for embedding **[1]**.~~

~~2.8.1. Talent transferring plant tissue samples to labeled glass vials.~~

3. LR-White Resin Embedding

3.1. To perfuse the samples, add increasing concentrations of LR-White resin in ethanol, beginning with 1 part resin to 3 parts ethanol. **[1-TXT]**. At each concentration, incubate for **[2]** 24 hours at 4 degrees Celsius **[3]**.

3.1.1. Talent adds resin in ethanol to glass vials. **TEXT: resin: ethanol series: 1:3, 2:3, 1:1, 3:2, 3:1, 1:0**
3.1.2. Talent places vial **1:3** in refrigerator
3.1.3. Added shot: talent Removes the last vial **1:0** from refrigerator.

3.2. Replace the LR-white resin with fresh resin, and incubate the samples for an additional 12 hours at 4 degrees Celsius **[1]**.

3.2.1. Talent replaces resin with new resin and places sample in refrigerator.

3.3. Prepare the embedding gelatin capsules, selecting capsules that are slightly larger than the samples, so that the samples can be completely enclosed in the resin **[1]**. Label paper tags with pencil, because ink will contaminate the resin **[2]**.

3.3.1. Talent selects capsules of appropriate size and opens them.
3.3.2. Talent labels paper tags.

3.4. Apply one drop of fresh LR-white resin to the bottom of each capsule **[1]**. Place a sample in each capsule. Then, fill the capsules to maximum capacity with fresh resin **[2]**. Put the cap on the capsule and press gently to seal it **[3]**.

3.4.1. Talent applies a drop of resin to the bottom of each capsule.
3.4.2. Talent places a sample in a capsule and fills the capsule with resin.
Videographer: This is one of the most important steps for viewers to see.
3.4.3. Talent puts the cap on the capsule and presses gently.

3.5. To polymerize the resin, cure the capsules at 58 degrees Celsius until fully hardened, around 24 to 48 hours **[1]**.

3.5.1. Talent places capsules in an incubator.

4. Sample Trimming and Sectioning

4.1. After peeling the gelatin capsule from the LR-White resin, place the resin block under the stereomicroscope **[1]**.

4.1.1. Talent places capsule under stereomicroscope.

4.2. Using a sharp razor blade, trim the LR-White block at a 45-degree angle to the sample **[1]**. Rotate the sample, and make a second cut at a 90-degree angle to the first **[2]**. Continue cutting in the same pattern to form a pyramid, with the apex of the pyramid directly above the sample's area of interest **[3]**.
4.2.1. LAB MEDIA: 60134_video_1.mp4. 00:09 – 00:18.
4.2.2. LAB MEDIA: 60134_video_1.mp4. 00:19 – 00:32.
4.2.3. LAB MEDIA: 60134_video_1.mp4. 01:30 – 01:41.

4.3. Then, begin removing fine slices perpendicular to the major axis, until the cutting surface reaches the sample **[1]**. The target sample should ideally be enclosed in a trapezoid shape **[2]**.

4.3.1. LAB MEDIA: 60134_video_1.mp4. 02:19 – 02:27.
4.3.2. LAB MEDIA: 60134_video_2.mp4. 02:28 – 02:31.

4.4. Place the trimmed resin block in the ultra-microtome **[1]**. Adjust the angle between the knife edge and the resin block **[2]**. Cut sections from the block with a thickness between 200 and 700 nanometers **[3]**.

4.4.1. Talent places resin block in microtome.
4.4.2. LAB MEDIA: 60134_video_2.mp4. 00:46 – 00:57.
4.4.3. LAB MEDIA: 60134_video_2.mp4. 02:05 – 02:15.

4.5. Use a wire loop to carefully lift some sample sections from the microtome **[1]**. Place them in a drop of distilled, deionized water on a glass slide **[2]**. Evaporate the water by placing the slide on a hotplate at 50 degrees Celsius **[3]**.

4.5.1. LAB MEDIA: 60134_video_2.mp4. 02:20 – 02:24.
4.5.2. Talent places sample sections in drop of water on glass slide.
4.5.3. Talent sets hotplate temperature and places slide on a hotplate.

4.6. Then, add a drop of stain to the sections **[1-TXT]**. After 30 seconds, rinse off the stain and observe the slide under the microscope **[2]**, to verify the general state of the section and that the desired plant structure is visible. **[3]**.

4.6.1. Talent adds a drop of stain to the slide. **TEXT: Stain: 1% Toluidine Blue O (w/v) in 1% boric acid solution (w/v)**
4.6.2. Talent rinses off stain and places slide on microscope stage.
4.6.3. SCOPE: Plant structure in stained slide.

4.7. Prepare a clean reaction slide by placing a drop of distilled, deionized water in each well of the slide **[1]**. Transfer one or two sample sections to each drop of water **[2]**.

4.7.1. Talent prepares reaction slide by placing a drop of water in each well.
4.7.2. Talent transfers sample sections to the slide. *Videographer: This is one of the most important steps for viewers to see.*

4.8. Place the slide in a 10-centimeter-square Petri dish, cover it, and let it dry at 50 degrees Celsius **[1]**.

4.8.1. Talent places slide on hot plate.

5. Immunolocalization

5.1. To prepare an incubation chamber for the reaction slides, place some dampened paper towels at the bottom of a pipette-tips box and wrap the box with aluminum foil **[1]**. Transfer the slides from the box to the incubation chamber **[2]**.

5.1.1. Talent places dampened paper towels in box and wraps box in foil.
5.1.2. Talent places slides in the incubation chamber (box).

5.2. Pipette 50-microliters of blocking solution into each well **[1-TXT]**. After incubating the slide for 10 minutes, remove the blocking solution. Then, wash all the wells twice with PBS, for 10 minutes each time **[2]**.

5.2.1. Talent uses pipette to add blocking solution to each well of reaction slide. **TEXT: Block solution: 5% nonfat dry milk (w/v) in 1 M PBS**

5.2.2. Talent removes blocking solution and begins adding PBS to the wells. *Videographer: This is one of the most important steps for viewers to see.*

5.3. After preparing the primary antibody solutions as described in the manuscript, perform a final wash of the wells with distilled, deionized water for 5 minutes **[1-TXT]**.

5.3.1. Talent washes the wells with water. **TEXT: Note: Never let the wells become completely dry.**

5.4. Pipette the primary antibody solution into the reaction wells **[1]**. Then, pipette the blocking solution into the control wells **[2]**.

5.4.1. Talent pipettes antibody solution into the reaction wells.

5.4.2. Talent pipettes blocking solution into the control wells.

5.5. Close the incubation chamber. Let it stand for 2 hours at room temperature **[1]**, and then refrigerate it overnight at 4 degrees Celsius **[2]**.

5.5.1. Talent closes the incubation chamber and the chamber is left on the lab bench.

5.5.2. Talent moves the incubation chamber to the refrigerator.

5.6. Prepare a 1 percent solution of the secondary antibody in blocking solution. Approximately 40 microliters per well will be needed **[1]**. Cover the solution with aluminum foil **[2]**.

5.6.1. Talent adds antibody to blocking solution.

5.6.2. Talent wraps the container of antibody solution in aluminum foil.

5.7. Wash the wells of the reaction slide twice with PBS and once with distilled, deionized water, for 10 minutes each time **[1]**. Ensure that no blocking solution or deposits remain in the wells **[2]**.

5.7.1. Talent begins washing slides with PBS.

5.7.2. ECU: Reaction slide, showing that no blocking solution or deposits remain in wells.

5.8. Pipette the secondary antibody solution into all the wells **[1-TXT]**. Incubate the slides in the dark, at room temperature, for 3 to 4 hours **[2]**.

5.8.1. Talent adds secondary antibody into all the wells. **TEXT: Protect slides from light.**

5.8.2. Talent places cover on pipette-tip box incubation chamber.

5.9. Again, wash the wells of the reaction slide twice with PBS and once with distilled, deionized water **[1]**. Then, add a drop of calcofluor white to each well **[2]**. Without washing, add a drop of mounting medium to each well **[3]**, and cover each well with a coverslip **[4]**.

5.9.1. Talent begins washing the wells with PBS.
5.9.2. Talent adds a drop of calcofluor to each well.
5.9.3. Talent adds a drop of mounting medium to each well.
5.9.4. Talent covers each well with a coverslip.

5.10. Observe the sample sections in the reaction slide using a fluorescence microscope **[1]**. For each observation, use a UV filter to detect cell walls stained by the calcofluor and a FITC filter to detect immunolocalization **[2]**. *Videographer: This is one of the most important steps for viewers to see.*

5.10.1. Talent places reaction slide on microscope stage.
5.10.2. SCOPE: Two stains ***or*** LAB MEDIA: Figure 1. *Video editor, show Figure 1I only.*

5.11. For a better visualization of the results, use ImageJ or a similar image analysis program to merge each UV-filter image with the corresponding FITC-filter image **[1]**.

5.11.1. SCREEN: 61034_screencapture_1.mp4. 1:34 – 1:44.

Results

6. Results: Subcellular Localization of Proteins and Pectins

6.1. By pinpointing the location of specific epitopes, this protocol enables characterization of the cell wall composition **[1]**. For example, 1,5-arabinan is abundant in the cell wall of the developing *Quercus (pronounce kwer-kəs) suber* anther **[2]**.

6.1.1. LAB MEDIA: Figure 2.
6.1.2. LAB MEDIA: Figure 2. *Video editor, show only Figure 2A.*

6.2. Scarcely esterified homogalacturonans are typically found at the root tip of *Quercus suber* embryo, specifying mechanical properties of the organ **[1]**. Xylogalacturonans are found in degenerating cells, such as the endosperm cells during the final stages of the *Quercus suber* acorn maturation **[2]**.

6.2.1. LAB MEDIA: Figure 2. *Video editor, show only Figure 2B.*
6.2.2. LAB MEDIA: Figure 2. *Video editor, show only Figure 2C.*

6.3. AGPs epitopes recognized by JIM13 or JIM8 are found on structures related to reproduction, such as cell lines related to microgametogenesis in *Arabidopsis thaliana* **[1]**, and the stigmatic papillae and micropyle of the Basal Angiosperm *Trithuria submersa* **[2]**.

6.3.1. LAB MEDIA: Figure 2. *Video editor, show only Figure 2D.*
6.3.2. LAB MEDIA: Figure 2. *Video editor, show only Figure 2E and Figure 2F.*

6.4. Common mistakes in implementing this protocol are usually easy to detect and identify **[1]**. When the washes are skipped or the reaction wells are allowed to dry, the secondary antibody will usually appear as a smear **[2]**.

6.4.1. LAB MEDIA: Figure 3.
6.4.2. LAB MEDIA: Figure 3. *Video editor, show only Figure 3A.*

6.5. Aggregates of green fluorochrome will form if the unbound primary antibody is not properly washed away **[1]**. Folding or detachment of the sections is usually related to poor adhesion, due to the use of unclean slides or aggressive washing **[2]**.

6.5.1. LAB MEDIA: Figure 3. *Video editor, show only Figure 3B.*
6.5.2. LAB MEDIA: Figure 3. *Video editor, show only Figure 3C.*

6.6. Sample preparation is also critical **[1]**. The resin blocks should be free of cracks, with a clearly visible, pale yellow to light brown sample **[2]**. Inefficiently embedded samples will show powdery white spots or areas **[3]**.

6.6.1. LAB MEDIA: Figure 3. *Video editor, show only Figure 3D.*

6.6.2. LAB MEDIA: Figure 3. *Video editor, show only Figure 3D, and emphasize Figure 3D-1.*

6.6.3. LAB MEDIA: Figure 3. *Video editor, show only Figure 3D, and emphasize Figure 3D-2.*

6.7. Keeping the sample size under 8 millimeters is essential for penetration of the fixative solution. Poorly fixed samples will appear dark brown or almost black **[1]**. Excessive temperature can cause the resin to crack, making sectioning of the sample impossible **[2]**.

6.7.1. LAB MEDIA: Figure 3. *Video editor, show only Figure 3D. Emphasize Figure 3D-3.*

6.7.2. LAB MEDIA: Figure 3. *Video editor, show only Figure 3D. Emphasize Figure 3D-4.*

Conclusion

7. Conclusion Interview Statements

Authors: Please memorize the interview statements prior to your filming day.

7.1. **Silvia Coimbra:** The use of immunolocalization techniques opens several research directions. One can follow on with more specific techniques such as biochemical analysis of the cell wall, or even proceed to a molecular approach. **[1].**

7.1.1. INTERVIEW: Named author says the statement above in an interview-style statement while looking slightly off-camera.

7.2. **Silvia Coimbra:** The results provided by this technique can help to understand the structure of the cell wall, an otherwise extremely complex structure very difficult to analyze by simple chemical analysis **[1].**

7.2.1. INTERVIEW: Named author says the statement above in an interview-style statement while looking slightly off-camera.