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TITLE:

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

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Arabinogalactan proteins; cell walls; Fluorescent immunolocalization; Histology; immunocytochemistry; LR-white resin embedding; Pectins; Plant Cell; Plant tissues.

SUMMARY:

This protocol describes in detail how the plant material for immunolocalization of Arabinogalactan proteins and pectins is fixed, embedded in a hydrophilic acrylic resin, sectioned and mounted on glass slides. We show cell wall related epitopes will be detected with specific antibodies.

ABSTRACT:

Plant development involves constant adjustments of the cell wall composition and structure in response to both internal and external stimuli. Cell walls are composed of cellulose and non-cellulosic polysaccharides together with proteins, phenolic compounds and water. 90% of cell wall compositions are polysaccharides (e.g., pectins) and arabinogalactan proteins (AGPs). The fluorescent immunolocalization of specific glycan epitopes in plant histological sections remains a key tool to uncover remodeling of wall polysaccharide networks, structure and components.

Here, we report an optimized fluorescent immunolocalization procedure to detect glycan epitopes from AGPs and pectins in plant tissues. Paraformaldehyde/glutaraldehyde fixation was used along with LR-White embedding of the plant samples, allowing for a better preservation of the tissue structure and composition. Thin sections of the embedded samples obtained with an ultra-microtome were used for immunolocalization with specific antibodies. This technique offers great resolution, high specificity, and the chance to detect multiple glycan epitopes in the same sample. This technique allows subcellular localization of glycans and detects their level of accumulation in the cell wall. It also permits the determination of spatio-temporal patterns of

AGP and pectin distribution during developmental processes. The use of this tool may ultimately guide research directions and link glycans to specific functions in plants. Furthermore, the information obtained can complement biochemical and gene expression studies.

INTRODUCTION:

Plant cell walls are complex structures composed of polysaccharides and glycoproteins. Cell walls are extremely dynamic structures whose architecture, organization and composition vary according to cell type, localization, developmental stage, external and internal stimuli[1]. Arabinogalactan proteins (AGPs) and pectins are important components of the plant cell wall. AGPs are highly glycosylated proteins and pectins are homogalacturonan polysaccharides whose composition, amount and structure vary greatly during different plant developmental stages[2-4]. AGPs and pectin studies have revealed their involvement in several plant processes such as programmed cell death, response to abiotic stresses, sexual plant reproduction, among many others[5]. Most of these studies started with information obtained from immunolocalization studies.

Given its complexity, the study of cell walls requires many different tools. Detection of glycan epitopes using monoclonal antibodies (mAbs) is a valuable approach to resolve polysaccharide and glycoprotein distribution along this structure. There is a large collection of mAbs available to detect glycan epitopes and the specificity of each mAb is continuously being improved as well[6]. The technique here described is applicable to all plant species, and is a perfect tool to guide future research directions that might involve more expensive and complex techniques.

In this technique, specific antibodies are chemically conjugated to fluorescent dyes such as FITC (fluorescein isothiocyanate), TRITC (tetramethylrhodamine-5-(and 6)-isothiocyanate) or several Alexa Fluor dyes. Immunofluorescence offers several advantages, allowing a clear and quick subcellular localization of glycans that can be directly observed under a fluorescence microscope. It is highly specific and sensitive, since the preparation of the sample can effectively protect the natural structure of the antigen, even if present in lower amounts. It allows the detection of multiple antigens in the same sample and most important, offers high quality and visually beautiful results. Despite the great power offered by fluorescence immunolocalization studies, they are often regarded as difficult to perform and implement most probably due to the lack of detailed protocols allowing the visualization of the different steps of the procedure. Here, we provide some simple guidelines on how to perform this technique and how to obtain high quality images.

For the protocol presented here, samples must first be fixed and embedded using the most appropriate fixative. Although considered as a time consuming and relatively tedious technique, proper fixation and embedding of the plant sample is the key to ensure a successful immunolocalization assay. For this purpose, the most usual is chemical fixation using crosslinking fixatives, like aldehydes. Cross-linking fixatives establish chemical bonds between molecules of the tissue, stabilizing and hardening the sample. Formaldehyde and glutaraldehyde are cross-linking fixatives, and sometimes a mix of both fixatives is used[7].

Formaldehyde offers great structural preservation of tissues and for extended periods of time, producing small tissue retractions and being compatible with immunostaining. Glutaraldehyde is a stronger and stable fixative usually used in combination with formaldehyde. The use of glutaraldehyde has some disadvantages that must be taken into account as it introduces some free aldehyde groups into the fixed tissue, which may generate some unspecific labeling. Also the crosslinking between proteins and other molecules occasionally may render some target epitopes inaccessible for the antibodies. To avoid this, the quantity and duration of the fixation must be carefully defined.

After fixation, samples are embedded in the proper resin to harden before obtaining the sections. London Resin (LR-White) acrylic resin is the resin of choice for immunolocalization studies. Differently from other resins, LR-White is hydrophilic, allowing the antibodies to reach their antigens, with no need of any treatment to facilitate it. LR-White has also the advantage of offering low auto-fluorescence, allowing a reduction in background noise during immunofluorescence imaging.

There are many staining techniques available to detect different components of the cell wall, such as Alcian blue staining, toluidine blue staining or Periodic acid–Schiff (PAS) staining. None of these offers the power of immunolocalization analyses[8]. This approach gives greater specificity in the detection of glycans, offering vaster information regarding cell wall composition and structure.

PROTOCOL:

1. Sample Preparation: fixation, dehydration, and LR-White embedding

1.1. Fixation and dehydration

NOTE: The fixation process is critical to preserve the sample; by crosslinking molecules the cellular metabolism is stopped, ensuring cellular integrity and preventing molecular diffusion. Fixative agents and the concentration used must be adjusted to that purpose, leaving the antigens sufficiently exposed to interact with the antibodies. The following protocol combines the mild fixative capability of paraformaldehyde, with the stronger effect of glutaraldehyde. Their proportions were optimized for AGPs and cell wall components, but it is suitable for other proteins and cell structures. The subsequent dehydration process will prepare the samples for LR-White embedding. Plant tissues from several plant species were used in this experiment. *Quercus suber* samples were collected from trees grown in the field. *Trithuria submersa* samples were kindly shared with us by Paula Rudall (Kew Gardens, London, UK).

1.1.1. Sow all Arabidopsis plants directly on soil and grow in an indoor growth facility with 60% relative humidity and a day/night cycle of 16 h light at 21 °C and 8 h darkness at 18 °C. Select the plant tissues to be analyzed, and trim samples to be no more than 16 mm^2 in size.

1.1.2. Immediately transfer the sample to a glass vial previously filled with enough cold fixative solution (2% formaldehyde (w/v), 2.5% glutaraldehyde (w/v), 25 mM PIPES pH 7 and 0.001% Tween-20 (v/v)) to completely submerge the samples (**Figure 1A**).

CAUTION: Formaldehyde and glutaraldehyde are both fixative agents that can be harmful by inhalation and contact. Perform the fixation step on a fume hood and wear adequate protective clothing and nitrile gloves. All solutions from this step onwards, including alcohol series until 70%, should be stored for later decontamination by specialized staff.

1.1.3. After gathering all the samples, refresh the fixative.

1.1.4. Transfer the vials to a vacuum chamber, and slowly apply vacuum. Upon reaching -60 kPa, the floating material will start to sink to the bottom of the vial (**Figure 1B**).

1.1.5. Keep under a vacuum of no more than -80 kPa for 2 h at room temperature.

1.1.6. Slowly release the vacuum, seal the glass vial and place overnight at 4 °C.

1.1.7. Discard any samples that did not sink during the overnight fixation. Wash the remaining samples with 25 mM PBS pH 7 for 10 min, followed by a 20 min wash in 25 mM PIPES buffer pH 7.2.

1.1.8. Dehydrate the samples in an ethanol series (25%, 35%, 50%, 70%, 80%, 90%, and 3x 100% ethanol) for 20 min each. Immediately transfer the dehydrated samples to labeled glass vials for embedding (**Figure 1C**).

NOTE: The dehydration process can be paused at the 70% ethanol step.

1.2. LR-White resin embedding

NOTE: LR-white is a non-hydrophobic acrylic resin with low viscosity, making it ideal for penetrating tissues with many thick cell walls layers. LR-White also comes in several hardness grades compatible for cutting most plant samples. Please do make sure that the used resin is already supplied with the proper catalyst mixed in, or follow the supplier instructions to prepare the resin. The following embedding process is slow but the results justify greatly the means.

1.2.1. Perfuse the samples by incubating with the LR-White resin in a series concentration of resin (1:3, 2:3, 1:1, 3:2, 3:1, 1:0) in ethanol, incubating for 24 h at 4 °C in each step.

CAUTION: LR-White resin is a low toxicity acrylic resin; however, it may be an irritant to the skin by contact and inhalation. Working in a well-ventilated area or under a fume hood with appropriate protective wear and nitrile gloves is highly recommended.

1.2.2. Refresh the LR-white resin and incubate for an additional 12 h at 4 °C.

1.2.3. Prepare appropriate size embedding gelatin capsules (size 1 (05 mL) for samples up to 3 mm, 2 x 0.37 mL for samples up to 5 mm or 3 x 0.3 mL for samples up to 8 mm, and paper tags (**Figure 1D**).

NOTE: Select the capsule size to be slightly larger than the sample, so that the specimen can be completely enclosed in the resin. Also, remember to label the tags with a pencil, because pen or printed inks will contaminate the resin, ruining the sample.

1.2.4. Apply one drop of fresh LR-white resin to the bottom of each capsule.

1.2.5. Place a sample in each gelatin capsule and fill to maximum capacity with fresh resin. Place the capsule cap and press gently to form a hermetic seal (**Figure 1E**).

1.2.6. Polymerize the resin for 24 to 48 h at 58 °C, or until fully hardened.

1.2.7. Store samples at room temperature.

NOTE: Post polymerization LR-white resin is inert.

2. Slide preparation

NOTE: Glass slides must be clean, free of any dust, grease or any other contaminants. Even new slides must be cleaned as some suppliers use oils and detergents to prevent the slides from sticking together. Any grease or detergent will interfere with the section addition to the slide, even if treated with poly-L-lysine. Lint and dust will affect the specimen's observations and very possibly ruin the experiment. Teflon coated slides with reaction wells are perfect for this task. They are affordable, reusable and drastically reduce the amount of antibody solution needed. With proper cleaning, excellent quality fluorescent immunolocalization can be performed at a very affordable cost.

2.1. Slide washing

2.1.1. Place the slides in a staining rack and cover with cleaning solution (0.1% SDS (w/v), 1% acetic acid (v/v), 10% ethanol (v/v)).

2.1.2. Maintain a mild agitation for 20 min.

2.1.3. Transfer the staining racks to a ddH_2O bath with mild agitation for 10 min. Repeat 4 times.

2.1.4. Carefully drain the racks before dipping briefly in 100% ethanol and let the slides dry in a dust-free environment.

2.1.5. Store until use.

2.2. Poly-L-lysine coating (optional)

NOTE: Small sections usually stick very well to clean glass slides. This step is only recommendable for larger sections (>2 mm^2). Larger sections tend to fold and wrinkle, and are not advisable. Nevertheless, if needed use reaction slides with larger holes. Clean them as above and proceed as follows.

2.2.1. Place clean slides in a square Petri dish. Pipette 0.001% poly-L-lysine solution (w/v) to cover the holes of the slides, without overflowing.

2.2.2. Let the slides dry overnight at 40 °C in closed Petri dishes.

NOTE: The coated slides are ready for immediate use and can be stored in a dust free environment at room temperature, for several months.

3. Sample trimming and sectioning

3.1. Sample trimming

3.1.1. With a sharp razor blade, trim the LR-White blocks under a stereomicroscope to form a pyramid shaped structure where the apex is perpendicular to the area of interest of the sample (**Supplemental Figure 1A**).

NOTE: The ultra-microtome specimen holder is an excellent tool to secure the block. If the device does not have a universal specimen holder, use the one most fit for round blocks.

3.1.2. Trim the pyramidal structure by removing fine slices of the excess resin perpendicularly to the pyramid major axis.

3.1.3. Proceed until the sample is reached forming the cutting surface. Within the cutting surface, the target sample should ideally be enclosed in a trapezoid shape.

NOTE: The resin block can be further trimmed at a slit angle to reduce the section surface area with a sharp blade (**Figure 1F**).

3.2. Semi-thin sectioning

NOTE: A microtome with glass knives will be used. The ability of the antibody to connect to the epitope will condition the immunolabeling reaction success. The hydrophilic nature of the LR-White resin will allow for good contact of the antibodies with the sample sections. Thinner sections will present fainter Calcofluor coloration that will be used to help locate the sections

and assist with the imaging process. The same holds true for other stains that may later be applied. Also excessive thickness will affect image acquisition quality. A good compromise for section thickness can be found between 200 and 700 nm, depending on the tissue characteristics. Mount the blocks tightly on the specimen holder of the ultra-microtome.

3.2.1. With an ultra-microtome, make sections with a thickness between 200 and 700 nm (**Figure 1G**).

3.2.2. Check the section area by transferring some sections to a ddH_2O drop on a glass slide. Place the slide on a 50 °C hot plate until water evaporates. Stain placing a drop of 1% Toluidine Blue O (w/v) in 1% boric acid solution (w/v) over the sections for 30 s. Rinse and observe under the microscope.

3.2.3. Upon finding the desired structure, transfer one or two sections to each drop of ddH_2O previously placed on each well of a clean reaction slide.

3.2.4. Place each slide in a closed clean 10 cm square petri dish and let it dry at 50 °C.

3.2.5. Store slides in a clean archive box until use.

4. Immunolocalization

NOTE: The fluorescent immunolocalization procedure relies on sequential use of two antibodies. The primary antibody is raised against a specific target antigen. The secondary antibody is raised specifically against the primary antibody and for fluorescent techniques is conjugated to a fluorophore (FITC in this specific protocol). The primary antibody will be used to detect the target antigen in the sample, and the secondary antibody will be used to mark the location where the primary antibody connected to the sample after washing off the excess of primary antibody. Controls are an important part of this assay and must always be performed to insure the accuracy of the observations. One well on the slide should be reserved for use as a negative control, where the primary antibody treatment will be skipped, and therefore no signal should be observed at the end of the experiment. A positive control must be included in the experiment by treating one well with an antibody which labelling is already known and certain. The positive control is used to confirm the secondary antibody labelling effectiveness and reaction conditions while the negative control tests the secondary antibody specificity.

4.1. Prepare an incubation chamber by placing some damped paper towels at the bottom of a pipette tips box and wrapping it with tin foil (**Supplemental Figure 1B-1E**). Place the slides in the incubation chamber.

4.2. Pipette a 50 µL drop per 8 mm well of blocking solution (5% nonfat dry milk (w/v) in 1 M PBS) and incubate for 10 min. Remove the blocking solution and wash all wells twice with PBS for 10 min.

4.3. Prepare the primary antibody solutions (see **Supplemental Table 1**), 1:5 (v/v) antibody in blocking solution; make an estimate of about 40 µL per 8 mm well.

NOTE: The concentration of the antibody must be adjusted according to the manufacture's protocol.

4.4. Perform a final wash with ddH_2O for 5 min, never let the wells dry completely.

4.5. Pipette the primary antibody solution to the reaction wells. Pipette blocking solution to the control wells.

4.6. Close the incubation chamber and let stand for 2 h at room temperature followed by overnight at 4 °C. Prepare the secondary antibody solution, 1% in blocking solution (v/v) about 40 µL per well. Keep it covered with tin foil.

4.7. Wash all wells twice with PBS for 10 min, followed by 10 additional minutes with ddH_2O. Make sure that no trace of the blocking solution or deposits is visible on the wells.

4.8. Pipette the secondary antibody solution to all wells. From now on, protect the slides from light.

4.9. Incubate for 3–4 h in the dark at room temperature.

4.10. Wash all wells twice for 10 min with PBS followed by another wash of 10 min with ddH2O.

4.11. Apply a drop of calcofluor (1:10,000 (w/v) fluorescent brighter 28 in PBS) to each well. Without washing, apply a drop of mounting medium (see **Table of Materials**) to each well and place a coverslip (**Figure 1H**).

4.12. Observe with a fluorescence microscope, equipped with 10x/0.45, 20x/0.75, 40x/0.95 and 100x/1.40 lens, use UV (for calcofluor stain) and FITC filters, to detect cell wall and immunolocalization respectively (**Figure 1I**). Use the following wavelengths: excitation/emission (nm) 358/461 for UV and 485/530 for FITC.

4.13. For a better visualization of the results overlap both images with ImageJ or similar.

REPRESENTATIVE RESULTS

In a successful experiment, the secondary antibody will specifically pinpoint the location of the specific epitope in bright green, in a consistent manner, allowing for the characterization of the cell wall composition at a certain development stage of the cell, tissue or organ. For example the LM6 antibody has an high affinity for 1,5-arabinan, a compound with type-I rhamnogalacturonan that can be found abundantly labelling the cell wall of the developing *Quercus suber* anther (**Figure 2A**), thus allowing to conclude that this type of pectin is abundant and part of the primary cell wall composition. JIM5 has affinity for homogalacturonans scarcely

esterified that are typically found at the root tip of *Quercus suber* embryo, specifying mechanical properties of the organ (**Figure 2B**). Xylogalacturonan are a type of pectin rich in xylose associated with cell wall loosening, they are found in degenerating cells. The antibody LM8 specifically recognizes xylogalacturonans, in maturing organs it may be used to detect degenerating cells or tissues, like the endosperm cells during the final stages of the *Quercus suber* acorn maturation (**Figure 2C**).

JIM13 has affinity for AGPs found on structures related with reproduction, cell lines related to microgametogenesis in *Arabidopsis thaliana* (**Figure 2D**). JIM8 also recognizes epitopes of AGPs present in cells and organs related to reproduction like the stigmatic papillae and micropyle of the Basal Angiosperm *Trithuria submersa* (**Figure 2E-2F**). Both antibodies have been suggested to be molecular markers for the gametophytic cell lines in plants[9].

Common mistakes to this protocol are normally easy to detect and identify. When the washes are skipped or the reaction wells are let to dry, the secondary antibody will usually appear as an unspecific smear covering indiscriminately over cells, tissues, resin and slide (**Figure 3A**). Aggregates of green fluorochrome will form if the unbounded primary antibody has not been properly washed away (**Figure 3B**). Another common cause for failure of this technique is related with the folding and/or detachment of the sections (**Figure 3C**), rendering the experiment useless. This problem is usually related with either poor adhesion of the sections to the slides, probably due to the use of unclean slides, and/or aggressive washing.

The sample preparation is also a critical step in this procedure. Fortunately, the most common fixative and embedding issues are easy to spot (**Figure 3D**). If all goes well the resin block will be free of cracks and the sample will be clearly visible with a pale yellow to light brown color (**Figure 3D-1**). Samples inefficiently embedded will show powdery white spots or areas (**Figure 3D-2**). Keeping the sample size under 8 mm is important to guarantee the penetration of the fixative solution. When samples are poorly fixed, they will appear dark brown almost black (**Figure 3D-3**). Also the temperature of polymerization is important for both the preservation of the epitopes and proper hardening of the resin. An excessive temperature can cause the resin to crack making the sectioning of the sample impossible (**Figure 3D** and **Figure 4**).

Figure 1. Overview of the complete protocol.

(**A**) For a successful fixation, the sample must be trimmed to a size under 16 mm^2 and immediately submerged in the fixative solution. (**B**) A mild vacuum (over -80 mPa) should be applied to ensure penetration of the fixative solution and remove any air pockets in the sample. (**C**) After fixation, the samples are dehydrated with a crescent series of ethanol concentrations; this step is essential for the success of the sample resin embedding. (**D**) Gelatin capsules are used as molds for the sample blocks. The gelatin capsules must be prepared and labeled with pencil marked paper strips that are rolled and inserted in the bottom part of the gelatin capsules. (**E**) The dehydrated samples are gradually embedded with a crescent concentration of acrylic resin and are finally transferred to the capsules labeled in (**D**). The capsules are placed in an oven at 58 °C for the resin to dry. (**F**) The hardened capsules are easily trimmed, with a sharp razor blade under a stereoscope, to remove the excess resin and expose the target sample. (**G**)

An ultra-microtome equipped with glass knives is used to make sections of the sample. Cut sections float over the water in turf feting the glass knife used to cut the sample. The floating samples are recovered with a wire loop. (**H**) Section containing tissues of interest are transferred to the wells of reaction slides where the immunolocalization procedure will be performed. (**I**) After the immunolocalization procedure, slides are observed under a fluorescent microscope equipped with a Green and UV filter, for visualization of the FITC labelled antibody and Calcofuor-white stain, respectively. Captured images of FITC labeled antibody (green) and Calcofluor-white stain (blue) can be merged for a better representation. Scale bar 100 µm.

Figure 2: Typical results of AGPs and Pectin immunolocalization in plant samples.
(**A**) LM6 labeling of arabinan moiety of pectins in a *Quercus suber* anther in meiosis I. (**B**) Specific labeling of low methyl-esterified pectins in a *Quercus suber* embryo root tip by JIM5. (**C**) Xylogalacturonan labeled by LM8 in the receding endosperm of a *Quercus suber* maturing acorn. (**D**) JIM13 labeling of AGPs in the tapetum and tetrads of an *Arabidopsis thaliana* anther. (**E**) AGPs labeled by JIM8 in the stigmatic papillae of *Trithuria submersa*. (**F**) JIM8 labelling AGPs at the micropyle (white arrow) of a *Trithuria submersa* ovule. Meiotic microspore (Mc), Tapetum (Tp), Root (R), Root tip (Rt), Testa (Ts), Endosperm (Ed), Embryo (Em), Epidermis (Ep), Stigmatic papillae (SP), Ovule (OV). FITC labeled antibody (green) and Calcofluor-white stain (blue). Scale bars: 100 µm.

Figure 3: Common mistakes and problems during the protocol.
(**A**) Failure of wash steps are identified by the presence of unspecific smear of secondary antibody (*) over sample and resin. (**B**) The poor specificity or wash failure of the primary antibody results in the formation of FITC aggregates (+) not bonded to a specific location. (**C**) Folds and section detachment are often the result of aggressive wash and unclean slides. (**D**) Examples of sample fixation and embedding; (1) perfect sample size and embedding, (2) embedding failure, (3) sample fixation failure, (4) resin hardening failure. FITC labeled antibody (green) and Calcofluor-white stain (blue). Scale bars: 100 µm.

Supplemental Figure 1. (**A**) Schematic representation of the block trimming sequence for preparing the sample (Yellow block) for sectioning with the ultramicrotome. Firstly, the gelatin capsule is removed (1). Then they are first trimmed at a 40° to 45° angle to the sides of the capsule tangentially to the sample (2), a second cut is made at 90° of the first (3) followed by a third (4) and fourth following the same rule (5). From this first phase of trimming results a pyramidal shaped structure which summit is located above the sample. Finally, with a sharp blade the summit is shaved off perpendicularly to the resin block major axis to reach the embedded sample. A square or trapezoid surface should be obtained at the end of the procedure (6). (**B**) Required supplies to make an incubation chamber; tin foil (1), double sided duct tape (2), paper towels (3) and a pipette tip box. (**C**) First step, the tin foil is fixed to the box with the double-sided duct tape. (**D**) Second step, the pipette tips holder is removed to place paper towels at the bottom of the box. (**E**) Third step, the paper towels are damped with water and the pipette tips holder is placed back to act has a tray for the slides.

Supplemental Table 1: List of useful monoclonal antibodies. The above table represents an example of available antibodies, with information about their targets and where they can be purchased.

DISCUSSION:

The fluorescent immunolocalization method in plants here described, while seamlessly straightforward, relies on the success of several small steps. The first of which is sample preparation and fixation. During this first step, a mixture of formaldehyde and glutaraldehyde is used to crosslink the majority of the cell components. The formaldehyde in the solution provides a mild and reversible fixation while the glutaraldehyde provides a strong more permanent linkage; the balance between the two fixatives provides the appropriate amount of crosslinking, allowing exposure of the epitopes to react with the primary antibody[10]. For the fixative solution to work properly, the sample must be small. Large structures more than 7 mm in diameter are very difficult to fix and should be clipped to ensure fixative penetration.

After wounding or stress, some plants secrete large amounts of tannins forming dark precipitates that may react with the formaldehyde interfering with the fixative process. Keeping the samples on ice and replacing the fixative solution whenever it becomes cloudy helps to reduce this effect. Air can also interfere with the fixative process and later on in the embedding and hardening of the resin. The proposed vacuum treatment should remove most of the air. For particularly airy tissues, the vacuum step may be extended further than 2 h, until no more air comes out of the samples and they sink to the bottom. Any sample found floating after the overnight fixative step should be discarded. Resin embedding provides support for cutting the preserved sample, and its hardness should be approximate to the embedded tissues[10]. LR-White comes in three hardness grades: hard for woody heavily sclerified tissues, medium grade for the vast majority of tissues from leaves to pollen grains, and soft grade for more delicate tissues. For a perfect embedding, the resin must completely penetrate the sample; this is better obtained with a slow and gradual infiltration. With prior testing, shorter incubation periods may be used. The gelatin capsules are both small and hermetically sealable, which makes them perfect for LR-White resin curing. For a size 2 (0.37 mL), curing should be complete after 24 h at 58 °C. For other capsule sizes, curing time should be adjusted. The LR-White cured resin should go from almost colorless to a light golden/amber color and feel hard to the nails. Sample trimming and the use of the ultra-microtome requires practice but will produce clear figures. The thickness and size of the section is important for the imaging and immunolocalization assay. The section thickness should be kept around 500 nm. Sections that are too thin will result in poor staining and section thicknesses over 700 nm will interfere with the image acquisition and resolution. The hydrophilic nature of LR-White resin allows for direct use of the sections in staining and immunolocalization without removing the resin.

The blocking solution (5% nonfat dry milk in 1 M PBS) reduces unspecific binding of antibodies. Nonfat dry milk has been a reliable alternative to BSA or other more traditional blocking agents, and is significantly less expensive. The blocking solution must be filtered through a paper filter prior to use, to avoid precipitates that form hard to wash away aggregates, retain antibodies, and compromise the experiment. The proposed antibody ratios and incubation times have

been optimized and successfully applied to diverse species in several studies[9,11-16].

Evaporation and exposition to light are two issues that must be avoided during the immunolocalization procedure. A humid and dark incubation chamber solves both of these problems. A tutorial on how to make a dark chamber can be found in **Supplemental Figure 1B-1E**. This immunolocalization protocol calls for the use of a primary antibody directed to the target epitope with no label of its own, and a secondary antibody conjugated to a fluorochrome (FITC) that is raised against the primary antibody IgG. This method offers several advantages over the use of a single antibody detection system due to an increased stringency of the detection, as the secondary antibody has no affinity to the target sample species. This system offers increased signal as the primary antibody molecules may be targeted by several molecules of the secondary antibody, each carrying a fluorochrome[17].

The decay of fluorochromes by intense light, or photo bleaching, is an important issue in this technique. Especially when exploring for faint localized signals, the signal may become irreversibly lost before imaging. Mounting medium greatly increases the stability and lifespan of the fluorochromes[18]; however, it is highly advisable to test the mounting media, as some may react with the stain and/or the fluorochrome, forming precipitates and blurring the image. The configuration of the optical system used for observing and registering the immunolocalization is one of the most important aspects for the success of this experiment. Due to the sections' reduced thickness, the benefits of using the confocal microscope are limited. The most successful setup requires a conventional upright epifluorescence microscope equipped with a fluorescent light source, LED or mercury light bulb[19], with adequate fluorescence grade objectives. Also good quality light filters set for excitation/emission (nm) 358/461 for UV and 485/530 for FITC are required. For the fluorescence image acquisition, refrigerated monochromatic digital cameras are recommended due to their high speed and sensitivity, but sacrifice true color information[20]. Polychromatic cameras provide the ability of easily sort out signal from background fluorescence but are slower and far less sensitive than their monochromatic counterparts.

The method is limited by the availability of specific antibodies. Despite the availability of a vast and expanding collection of antibodies aimed at plants epitopes, understandably not all plant compounds are yet covered. Also lipids tend to be extracted by the described embedding method, and is therefore not recommended for tissues with a high oil content. Cryostat section may be an alternative, despite sacrificing image resolution. The temperature of LR-White resin polymerization may in some cases alter more sensitive epitopes. If the target epitope is temperature sensitive, switch LR-White for LR-Gold resin. Polymerizing at -25 °C under white light, LR-Gold offers an excellent preservation of the thermosensitive epitopes, but will require the acquisition of a specialized polymerization apparatus and is slightly more toxic then LR-White.

This method has been used across a broad range of species and tissues. It allows fast access to reliable information on specific epitope distribution. Offering supporting data for evolutionary models and identifying markers and specific adaptations in cells and tissues while providing

visually enticing results. The use of antibodies conjugated with fluorochromes over peroxidase or alkaline phosphatase conjugation alternatives[10], is less time consuming, significantly less prone to overreaction artefacts and provides a clear subcellular resolution hard to match with any other technique. The use of antibodies specific to cell wall related polymers allows an insight into the arrangement of compounds into very restrict domains. Such resolution would be challenging to obtain from traditional biochemical tools. The ever-expanding set of primary antibodies available offers constant new discovery opportunities into the plant cell inner workings.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors declare no conflicts of interest.

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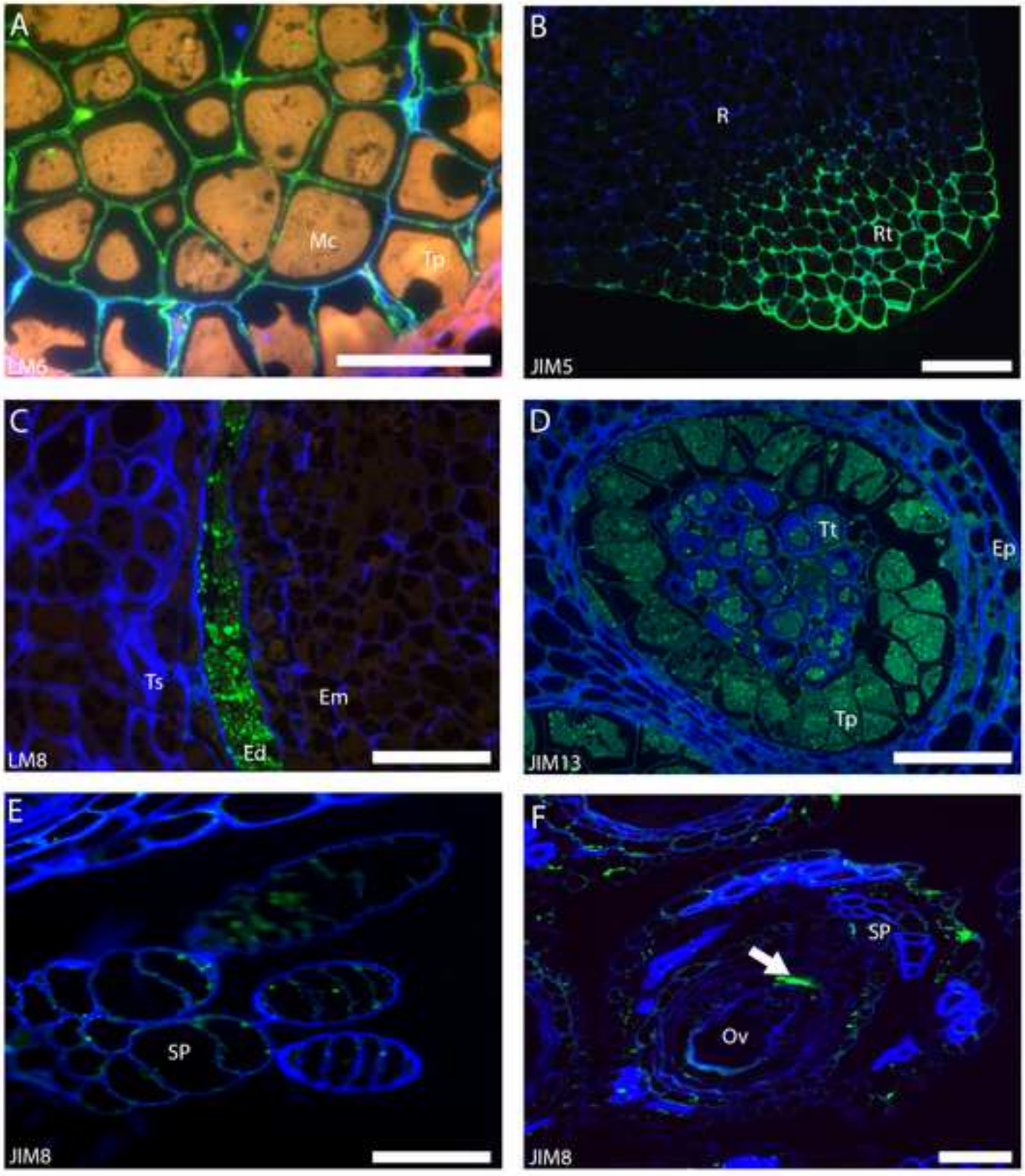
Figure

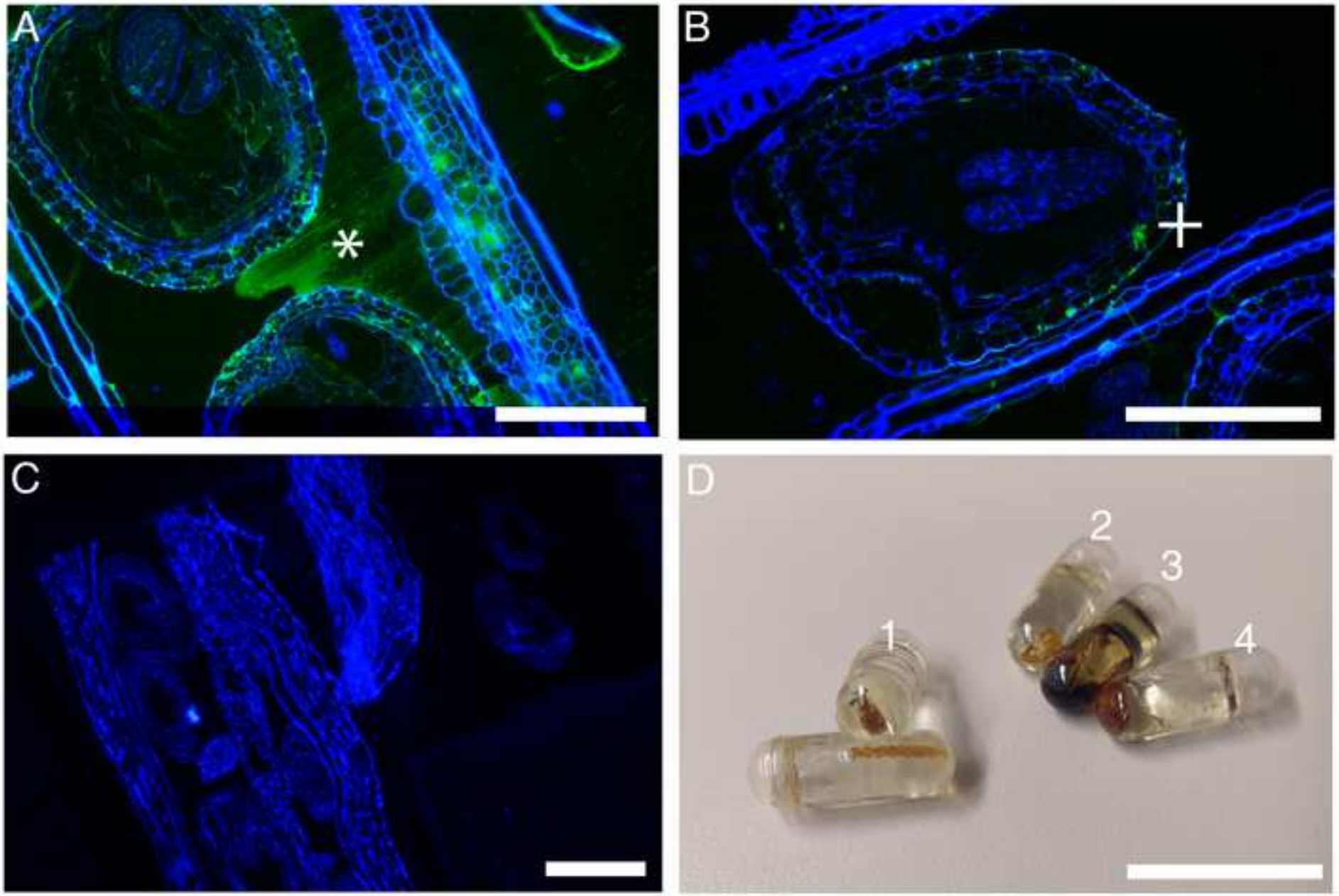
Figure

Click here to access/download;Figure;Figure 2.psd



Figure

Click here to access/download;Figure;Figure 3.psd



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
25% (w/v) Gluteraldehyde	Agar Scientific	AGR1010	aq. Solution, methanol free
8 wells Glass reaction slides	Marinfeld	MARI1216750	other brands may be used
Acetic acid	Sigma-Aldrich	A6283	
Anti-Rat IgG (wole molecule)-FITC antibody produce in GOAT	Sigma-Aldrich	F6258	
cover slips, 24 mm x 50 mm	Marinfeld	MARI0100222	The cover slip should cover all the wells. Other brands may be used
ddH_2O	na	na	
Ethanol absolute	na	na	
Fluorescent brightner 28	Sigma-Aldrich	F-6259	
Gelatin capsules	Agar scientific	AGG29211	The capsule size sould feat the size of the sample.
Glass vials	na	na	Any simple unexpensive glass vials that can be sealed, may be used. The vials may be clean with 96% etanol after use to remove LR-White residue and reused.

LR-white medium grade, embdeding resin	Agar Scientific	AGR1281	LR-White comes in several forms the medium grade provides na adequate cutting suport for most tissues for harder tissues a harder grade of LR-white may be recomendable. If possible use a resin already mixed with the polimeration activator (benzoyl peroxide), if not please folow the instructions of the suplier to prepare the resin.
Non fat dry milk	Nestlé	na	any non fat dry milk is adequate
Oven	na	na	generic laboratory oven
Petri dish, 10 cm x 10 cm square	na	na	
PIPES	Sigma Aldrich	P1851	

Rat generated Monoclonal Anti-Body	Plant probes	na	Several antibodies that recognize cell wall components are available at both the Complex Carbohydrate research center (CCRC, Georgia University USA) and Plant Probes (Paul Knox Cell Wall Lab, at Leeds University UK). A short list of some commonly used MABS and where they can be purchased is presented in Supplemental Table 1
Razor blades	na	na	regular razor blades
SDS	Sigma-Aldrich	L6026	
Toluidine Blue-O	Agar Scientific	AGR1727	
Tween 20	Sigma-Aldrich	P9416	
Ultramicrotome	Leica Microsystems	UC7	
upright epifluorescence microscope with UV and FITC fluorescence filters	Leica Mycrosistems	DMLb	
vaccum chamber	na	na	
vaccum pump	na	na	

Vectashield	vecta Labs	T-1000	Other anti-fade may be used. Please do check for compatibility with FITC and the Fluorescente brightner 28. (Note: for a non-commercial alternative, (see Jonhson et al 1982[18]) An antifade medium can be made by mixing 25 mg/mL of 1,4-Diazobicyclo-(2,2,2)octane (DABCO) in 9:1 (v/v) glycerol to 1xPBS. Adjust pH to 8.6 with diluted HCl.)

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06.01.2020

Revision of manuscript #JoVE61034

Dear Dr. Alisha DSouza,

Please find attached our revised manuscript entitled "Fluorescent Immunolocalization of Arabinogalactan proteins and pectins in plant tissues sections." by Mário Costa, Ana Marta Pereira and Sílvia Coimbra. The comments made by the reviewers were helpful in preparing a revision of the manuscript and we appreciate the time and effort invested by the referees. We have carefully addressed each comment and suggestion made by the reviewers. We hope that our manuscript will now be suitable for publication in JoVE, and look forward to hearing from you.

Yours sincerely,

Mário Costa

Answers to Editorial Comments:

Comment 1: Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

Answer: We've have done the requested proofreading carefully.

Comment 2: Abstracts: Please re-word the Summary to more clearly state the goal of the protocol. For example, "This protocol/manuscript describes…"

Answer: The Summary has been changed accordingly.

Comment 3: Textual Overlap: Significant portions show significant overlap with previously published work. Please re-write lines 110-117, 141-143, 161-165,205-213, 225-232, 252-256, 315-317, 326-329 to avoid this overlap.

Answer: All texts were modified accordingly.

Comment 4: Protocol Language: Please ensure that ALL text in the protocol section is written in the imperative voice/tense as if you are telling someone how to do the technique (i.e. "Do this", "Measure that" etc.). Any text that cannot be written in the imperative tense may be added as a "Note", however, notes should be used sparingly and actions should be described in the imperative tense wherever possible.
1) For example lines 110-117 should be a note.
2) For example lines 141--144 should be a note.

Answer: We've carefully checked all the text and changed accordingly.

Comment 5: Protocol Detail: Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Some examples:

Comment 5.1: 1.1.1: Please add plant strain, age etc. How were the plants grown and maintained? Please add this briefly. Also mention examples of tissues you analyzed. What are the dimensions of the samples? 8 mm x 8 mm x 8mm ? A weight reference will help.

Answer: Plant material details were added. We must remind you that this protocol is meant for application in a large variety of species and plant tissues. We have successfully applied this technique to a wide range of samples, from seeds to roots and leaves from very diverse plant species, this is the reason why no major details about plant material growth and maintenance was not added before, once different plant species will have different needs. We intend to provide here a protocol that works well for the majority of samples. That is one of the reasons for the maximum sample volume restriction rule of 8mm, as above this limit fixative will not be efficient for some harder samples, despite the weight of the sample.

Comment 5.2: 1.1.2: How much fixative?

Answer: The volume of fixative to be used will depend on the used vial capacity. The most important aspect of this step is for the sample to be totally submerged in the fixative solution. The text was changed accordingly.

Comment 5.3*: 1.1.3: What is the composition of the fresh solution? Or do you simply mean "refresh the fixative"?*

Answer: Yes, that was exactly what we meant. We have changed it accordingly, thank you for the suggestion.

Comment 5.4: 1.2.3: Mention size specifications.

Answer: The gelatin capsule size will depend on the shape characteristics of the sample, we believe that user should choose the one that best fits its needs. Changed accordingly.

Comment 5.5: 4.5: Mention antibodies and concentrations.

Answer: This protocol in not intended to be used with one single antibody, a wide selection of previously successfully used antibodies is made available for the reader in supplementary Table 1, also a note was introduced to clarify the need to adjust the concentration of the working solution according to the manufacturer recommendations.

Comment 5.6: 4.12: Mention lens magnification and N.A.

Answer: Thank you for your observation, however the aim of this protocol is that it can be used as widely as possible. Depending on signal strength, fluorescence may be captured successfully at low magnifications with a wide range of setups in an upright

epifluorescence or inverted microscope. Therefore, we fill that enforcing the idea that only a specific setting will work with this technic is not a requirement and can potentially be misleading.

Comment 6: Protocol Numbering: Please add a one-line space after each protocol step.

Answer: Changed accordingly.

Comment 7: Protocol Highlight: After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is a 10-page limit for the protocol text, and a 3- page limit for filmable content. If your protocol is longer than 3 pages, please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.
1) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
2) Notes cannot be filmed and should be excluded from highlighting.

Answer: changed accordingly.

Comment 8: Discussion: JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

Answer: This has been cautiously checked.

Comment 9: *Figure/Table Legends:*
1) Please expand the legends to adequately describe the figures/tables. Each figure or table must have an accompanying legend including a short title, followed by a short description of each panel and/or a general description.
2) Define the scale bars and pseudocolors.
3) Add legends and in-text references to supplementary figures and tables.
4) Remove legends from the figure files.

Answer: All the above issues have been appropriately checked and changed.

Comment 10: References:

1) Please make sure that your references comply with JoVE instructions for authors. Citation formatting should appear as follows: (For 6 authors or less list all authors. For more than 6 authors, list only the first author then et al.): [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage, doi:DOI (YEAR).]
2) Please spell out journal names.

Answer: All the above issues have been appropriately checked and changed.

Comment 11: Commercial Language: JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are Vectashield®,
1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.
2) Avoid product references in Suppl Table 1

Answer: All the above issues were addressed and changed in accordance.

Comment 12: Table of Materials: Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials/software in separate columns in an xls/xlsx file. Please include items such as antibodies and RRIDs

Answer: All the above issues were addressed and changed in accordance.

Comment 13: If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

Answer: All tables and figures used are original, and have not been published before.

Answers to Peer-Reviewers:

Reviewer #1:

*The manuscript **JoVE61034** describing the immunofluorescence method of cell imaging using molecular probes - specific antibodies against cell wall glycans has some little shortcomings. Please refer them for improvements of the manuscript. Below I point out the main remarks:*

Comment 1: Sample preparation and resin embedding: In my opinion, the part about the type of the fixation solution is too short and not scrupulously prepared. Without well-prepared research material, we will not get correct results. More information about the selected reagent should be added. Furthermore, for isolation of embryological structures such as anthers/ovules, we also may use a mixture of 2.5% paraformaldehyde and 2.5% glutaraldehyde in cacodylate buffer (pH 7.2) for 24 h at room temperature. The Authors should underline the way of preparation of the resin working solution and mention the addition of benzoyl peroxide. Without benzoyl peroxide, the polymerization process will not occur.

Answer 1: There is a wide variety of protocols that work well for preparing plant tissues, some more efficient than others depending on the nature of the sample. And indeed, we would love to include and discus them all. But, unfortunately we are constricted by the imposed page limits. Here we tried to present a protocol that is both simple, sufficiently robust and that exposes the user to a minimum of hazardous compounds.
Regarding resin preparation, in fact, the LR-white resin from our supplier, already comes mixed with the catalyst. However, some suppliers sell the resin and the catalyst separately. A note was included in the main manuscript and in the materials section to avoid this mistake to occur.

Comment 2: Slides preparation: Covering is an extremely important and necessary step which prevents washing the slices from glass. We can choose also organosilane polymers to cover microscope slides. However, today we can chose an easier way and buy a Poly-L-Lysine slides ready for use.

Answer 2: Yes indeed, the use of Poly-L-Lysine coated slides is widely spread and very useful. Still those slides are of single use, and also reaction chambers designed with either PAP or Dako pen are usually made bigger than needed, creating unnecessary waste of material and reagents. By suggesting the use and reuse of Teflon coated slides with predesigned reaction wells, we aim at reducing waste, and making the procedure simpler. As stated in the abstract, it is not the aim of this protocol to

present all the possible ways of performing this technique, but rather present our optimized protocol for this technique.

Comment 3: Sectioning: In this part of the procedure, the Dako pen is commonly used. It is a marking pen that provides a thin film when a circle is drawn around a specimen on the slide. This circle provides a water-repelling barrier to liquids, such as antibody solutions.

Answer 3: The Dako and PAP pen are useful and versatile tools. But, the area of the drawn circles are difficult to estimate, and a high level of experience is required to properly estimate the volume of solution to be used, as pointed out in the previous answer. Less experienced users will tend to overestimate the needed volume generating waste. By using the Teflon coated slides the circle size is predetermined making it possible to confidently estimate volume and make precise calculations to avoid waste. Once again, here we present one way of performing this method, there are many other options available.

Comment 4: Immunolocalization procedure: The concentration of particular antibodies such as 1:5 for primary antibodies is not a rule - we have to prepare the concentration according to the producer's protocol. To preserve fluorescence, tissue slices should be mounted onto microscope slides with Dako Fluorescent Mounting Medium to reduce fading of immunofluorescence during imaging.

Answer 4: Yes, we do agree with the reviewer's view, and a note was introduced in the protocol to advise the readers regarding antibodies concentration.
Regarding possible losses of fluorescence signal, we do propose the use of an anti-fade mounting medium. Vectashield is the European alternative to Dako (that is not available in Europe). A non-commercial alternative was also included in a note to the protocol.

Comment 5: Description of antibodies: Nowadays, we can choose also different hybridoma supernatants for a range of JIM-designated anti-arabinogalactan-protein (AGP) and anti-pectin rat monoclonal antibodies. The list of antibodies is not accurate: there is a lack of LM30, LM14, LM13, LM18, and LM26 available at Plant Probes (Leeds, UK) and JIM15&16 from CCRC (Georgia, USA).

Answer 5: Our supplementary table 1 was meant to be merely indicative of the abundance of available antibodies, and where to look for them, and not an exhaustive inventory.

Comment 6: More relevant studies should be included. Here I propose some of them but the list should not be limited to them:

* Wilson SM, Bacic A. 2012. Preparation of plant cells for transmission electron microscopy to optimize immunogold labeling of carbohydrate and protein epitopes. Nature Protocols 7(9), 1716-1727
* Bergersen LH, Storm-Mathisen J, Gundersen V. 2008. Immunogold quantification of amino acids and proteins in complex subcellular compartments. Nature Protocols 3(1), 144-152.

Answer 6: We greatly appreciate and value the recommendations of the reviewer, but the suggested references refer to the preparation of samples for electron microscopy, which is not the main purpose of the protocol here described. Yet they present very useful insight into the fixative process, and sample preparation. Thank you for your suggestion, we have adjusted the references in accordance.

Comment 7: The authors should revise the entire manuscript considering these points. Overall, due to the high importance of the research conducted with molecular tools and the difficulty of this method, the manuscript/protocol should be improved and then accepted for publication.

Answer 7: All the issues raised by reviewer #1 were carefully checked and addressed.

Reviewer #2

Manuscript Summary:

Useful protocol for research on plant cell wall composition, structure and remodeling by the sensitive technique of fluorescent immunolabeling. As the cell wall is a complex and dynamic structure of plant cells, the protocol can be applied to other components; the only limitation, as the authors point out, is the availability of specific antibodies. The list of equipment is extensive. The steps are well explained although the photos which illustrate the different steps should be taken closer. The controls are well listed and the most critical steps are highlighted. Nice results, including from the authors group were included to confirm the usefulness of the technique.

Major Concerns:

Title: introduce "cell wall"

For a better view some figures should be taken closer: Fig 1B, D, E (the size of Fig 3D is good), F, H

l.165 Teflon coated reaction slides... introduce here the concept of "wells" used thereafter

l. 236 Introduce in 4.1 the text of the legend of suppl. Fig. 1B-E

l. 296 ...color (Fig3D,1)

l. 296 .. 1reas (Fig 3D,2)

l. 298 ... black (Fig. 3D,3)

l. 301... impossible (Fig 3D,4)

Minor Concerns:

14-15 Key words different from l.46-l.47 Key words.

Introduction

l.61, 63, 66 repeating "this" makes reading not fluent

l. 126 specialized staff.

l. 184 ...as

l. 194 sharp

l. 342 ... milk has...

l. 350 your own...

l.362 18 superscript

l. 369-370 - rephrase for clarity

Answer to reviewer #1: We really appreciated the comments and revisions suggested by reviewer #2. Thank you for taking the time to carefully read our manuscript. All concerns were addressed, and changed accordingly.

Reviewer #3

Manuscript Summary:

All in all, I need the uahtors to explain to me the novelty of the methods they describe. I fail to see the novelty and need more info on why this should be published other than describing the methods in more detail than normal for a publication. Which is fine, if this is the iam. But this aspect could be clearer and I think they need to acknowledge pioneers in the field more. I think a major revision is in place bearing these points in mind.

Major Concerns:
not sure if the authors cite the proper papers, I miss many references here

Minor Concerns:
some language issues. e.g. what does "vaster" in line 104 mean?

Answer 1: We are aware that there is no novelty in this protocol, and we do not want that kind of credit. As we state in lines 35-36 of the Abstract: “Here we report an optimized fluorescent immunolocalization procedure to detect glycan epitopes from AGPs and pectins in plant tissues.” We aim to present a detailed protocol optimized by us in our laboratory, in more detail, to help researchers take full advantage of this technique during their investigations. Since this is a long protocol many times viewed as boring and difficult to implement, especially by researchers who do not use this technique routinely, we aim at taking advantage of the video protocol to show the readers that this can be easily made by any molecular biologist, e.g., and we aim at guiding them through all its tricky steps.
In line 104 by “vaster” we mean that the results given by this technique will offer the researcher more results than just localize an epitope, such as rich information about cell wall composition and structure.

Reviewer #4:

This paper describes in detail methodology for fixation, embedding, sectioning and immunostaining of plant tissues, with a particular emphasis on using plant cell wall glycan-directed monoclonal antibodies. These methods are well-established and well-used in the plant cell wall literature, though I am not aware of any video publication that presents these methods.
There are some issues with the manuscript that I think need to be addressed prior to publication:

Comment 1: The authors make claims to novelty in their manuscript that I think are not warranted. Their basic approach is used by quite a few laboratories, including my own. Indeed, there have even been detailed experimental methods papers published (but not cited by the authors), including Lee & Knox (2014) and Avci et al. (2012). Further, there is a comparative review of various methods for immunolabeling of plant tissues that has also not been cited by the authors (Verhertbruggen et al., 2017). And there may be others that I am not aware of. None of the methodological steps described by the authors are novel, though perhaps some of the specific details vary from published protocols. What is novel, as stated above, is the video publication. Others use mixed formaldehyde/glutaraldehyde fixatives, ethanol dehydration, LR White embedding, milk as blocking reagent, etc. So the authors need to careful about claims to novelty in their protocol.

Answer 1: We are aware that there is no novelty in this protocol, and we do not want that kind of credit. As we state in lines 35-36 of the Abstract: “Here we report an optimized fluorescent immunolocalization procedure to detect glycan epitopes from AGPs and pectins in plant tissues.” We aim to present a detailed protocol optimized by us in our laboratory in detail, to help researchers take full advantage of this technique during their investigations.

Comment 2: On line 62-63: The cited reference (Pattathil et al., 2010) IS appropriate with reference to the large collection of antibodies currently available. However, it is NOT appropriate with reference to the detailed binding specificity studies that have been done on a large percentage of the current antibody toolkit. More appropriate references would be Puhlman et al., Steffan et al., Clausen et al., Schmidt et al., and especially Ruprecht et al. (2017), where detailed studies of antibody binding specificities for plant glycan-directed antibodies have been reported.

Answer 2: Thank you for your helpful suggestion. References have been changed accordingly.

Comment 3: Lines 95-100: The authors need to be a bit careful here. It is true that LR White is a more hydrophilic embedding resin than other types of resins, and I agree that it is more suitable for immunolabeling studies. However, the authors should be clear that antibodies cannot penetrate the section and especially not the resin after polymerization. Labeling by the antibodies is a surface phenomenon. Antibodies can only label epitopes exposed on the cut surface of the section. The LR White facilitates access of the antibodies to the surface by not imposing a hydrophobic environment onto the surface of the section.

Answer 3: Thank you for pointing that out. The note inserted above that point in the protocol brings more clarity on that subject.

Comment 4: The article would benefit from editing by a native speaker of English to remove grammatical and typographical errors (e.g., l. 126: personnel instead of personal) and some awkward phrasings (though overall, the article is well-written).

Answer 4: We have carefully checked the text for this kind of mistakes, thank you for the suggestion.

Comment 5: All % solutions should provide reference as to the type of percentage being referred to (e.g., v/v, w/v, etc). In line 146, it is also not explicitly clear that the ratios refer to resin:ethanol ratios.

Answer 5: Adjustments in the text where made accordingly.

Comment 6: L. 225-235: The authors' reference to the importance of both negative AND positive controls is well taken and important. However, I would advise that the authors make it clear that the choice of a positive control antibody will depend on the material being sectioned. JIM7, the antibody that the authors mention as a positive control, does NOT label all cell walls in all plant tissues (for example, labeling with this antibody in monocot grasses will be quite sparse). I would advise the authors to state that the choice of a positive control antibody will depend on the tissue being examined and needs to be determined empirically, rather than making a specific recommendation.

Answer 6: Thank you for noticing that inaccuracy, the text was adjusted accordingly.

Comment 7: L. 293-301: Another factor that may influence the outcome of immunostaining procedures is the ethanol series used to dehydrate the samples during fixation and embedding. For some tissues, it may be advisable to use a longer

more gradual series of ethanol washes to better avoid tissue collapse that frequently happens during the dehydration step.

Answer 7: Yes, the reviewer is correct. Here we tried to present a method that has been successfully implemented with positive results with many different types of samples. Certainly, some adjustments must be made for samples that are more difficult.

Comment 8: L. 304-313: The authors need to be aware that the formaldehyde/glutaraldehyde fixatives will have little if any impact on glycans, as there are typically no functional groups in glycans that can react with these aldehydes (amino sugars are the exception, but these are in very low abundance in plant glycans). Thus, the purpose of the fixatives is to improve the overall structural stability of the sections by cross-linking the other polymers present (especially proteins!).

Answer 8: Once again we thank the reviewer for pointing out this important detail, adjustments have been made in the text to emphasize this aspect of the fixative effect.

Suplementary Table 1

Name	Affinity	Available at
LM2	Arabinogalactan protein	Plant probes (Leeds, U.K)
LM5	Pectins	
LM6	Arabinan	
LM7	Homogalacturonan	
LM8	Xylogalacturonan	
LM13	(1-5)-a-L-ARABINAN	
LM14	Arabinogalactan protein	
LM16	Processed Arabinan - RG-I	
LM18	Homogalacturonan	
LM19	Homogalacturonan	
LM20	Homogalacturonan	
LM26	BRANCHED-GALACTAN	
LM30	Arabinogalactan protein	
JIM5	Low methyl-esterified homogalacturonan	CCRC (Georgia, USA)
JIM7	Highly methyl-esterified homogalacturonan	
JIM4	Arabinogalactan protein	
JIM8	Arabinogalactan protein	
JIM13	Arabinogalactan protein	
JIM15	Arabinogalactan protein	
JIM16	Arabinogalactan protein	
JIM20	Extensin	
MAC207	Arabinogalactan protein	

Supplemental File (Figures, Permissions, etc.)

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