Practical Aspects of Immunomicroscopy on Plant Material

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Immunomicroscopy is a valuable method for studying the spatial and temporal expression and localization of biological molecules both by light and electron microscopy. Although immunolocalization is a well-established method, it is often considered as difficult when handling plant material. Here we discuss some practical aspects of immunomicroscopy, which should be helpful for a beginner to start with. Special focus is on the fixation and embedding plant material with LR White resin, and on the quality of antibodies and different controls to be used together with immunolabelling.

Keywords immunolocalization; plant; barley; fixation; antibodies

1. Introduction

There is an increasing need for immunolocalization studies on plant and plant-based materials. Comparing gene expression products in wildtype and mutant or transgenic plants, either in model plants such *Arabidopsis* [1], or in commercially important crops [2, 3] or woody plants [4], is often necessary. Studies on plant-defence mechanisms gain especially from immunomicroscopy [5]. Information of tissue and subcellular localization of molecules is useful in plant physiology and plant breeding as well as in studies considering the quality of crop plants [6]. More recent applications of immunomicroscopy include comparisons of plant-based raw material and processed products as one aspect of product development in food [7], and even textile and forest industry. Sufficient knowledge of the anatomy and cell types of plant tissues is necessary in all these applications in order to be able to interpretate the results.

There are several ways to perform immunolocalization and a beginner may feel lost between all the choices. The easiest way is to keep it simple at the beginning, for example testing if the already existing embedding method at the laboratory functions with the material. One thing to consider at the beginning is if immunolight microscopy is enough or if one is aiming for subcellular localization using electron microscopy. If light microscopy is enough, more simple protocol can be chosen. In any case, it is advisable to test the method at light microscopy level first and try to get as much information from that before proceeding to electron microscopy.

The most common method used in immunomicroscopy of plant material is chemical fixation coupled to resin embedding. Even though high-pressure freezing and subsequent freeze-substitution [8], or chemical fixation coupled to cryosectioning [9], are excellent methods for immunolocalization, the more conventional combination of chemical fixation and resin embedding may have some advantage for various reasons. Basic (electron) microscopy laboratory equipment is sufficient. The same sample blocks can be used for both light and electron microscopy, which may be particularly useful if studying plant/pathogen interactions. Resin blocks allow relatively large sample area which gives the possibility to choose the area of interest for ultrathin sectioning. The blocks will hold for years and can be stored at room temperature.

There are a number of embedding media suitable for immunomicroscopy [10, 11]. In the following, we have chosen to discuss how to handle plant material for chemical fixation and embedding with London Resin White (LR White), a relatively easy-to-use acrylic resin, and prerequisites of subsequent

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immunolocalization. We focus on practical aspects and relatively simple solutions which we consider helpful for a beginner.

2. Handling of plant material

Pretreatment of material to be used in immunomicroscopy requires careful planning beforehand. There are a number of questions: Which organs and tissues are of interest? Are there different treatments or timepoints to be compared? Are crossections or longitudinal sections preferred? How many samples are needed? It is easier to compare samples which are taken in a similar way from the beginning. It is best to try to limit the number of samples to be compared by screening the material with some other method(s) first, such as immunoblotting (western), immunotissue printing, dot blotting or enzyme activity measurement. A pilot study may be done in order to test the method and to get familiar with the procedure. For optimizing the method, one should choose material where the antigen is known to be abundant. Together with the positive tissue sample, one should have a negative tissue control, i.e. sample which does not contain the antigen or where the antigen is much lower in amount than in the positive sample.

2.1 Fixation

The purpose of fixation is to stop the metabolism, prevent diffusion of molecules and to create good preservation of structure in the tissues [11, 12]. It is a crucial step for any microscopy study, and thus, it is important to perform carefully. After fixation, one can either proceed directly to the following steps, or the samples can be kept in buffer at 4°C for later processing. This makes it possible to collect samples at different timepoints until further processing. Another practical aspect is that one can take for example half of the fixed tissue pieces for further processing, and leave another half in buffer just in case something would go wrong. For example, one can take ten tissue pieces of a sample, leave 5 in buffer and take 5 for further processing, and in the end, section 3 sample blocks.

Fixation for immunolocalization is always a compromise between preservation of antigenicity and good ultrastructure [12, 13, 14]. Fixation and subsequent treatments cover part of the antigenic epitopes available in the tissue thus decreasing the antigenicity. Aldehyde fixatives cross-link proteins, nucleic acids and fosfolipids thus forming a network which keeps the molecules in their original place. A combination of 4% paraformaldehyde and 0.05-0.25% glutaraldehyde functions well for immunomicroscopy of plant material. Paraformaldehyde is a mild fixative and penetrates tissues easily, which is an advantage with cell wall-containing plant tissues. Glutaraldehyde, on the other hand, is a stronger fixative with its two aldehyde groups and penetrates tissues slowly. It is necessary for good preservation of ultrastructure which is needed for electron microscopy studies. Osmium tetroxide is extremely powerful fixative and is not recommended for immunolocalization studies (unless the antigen is extremely abundant). According to our experience, 100 mM phosphate buffer at pH range 7.0-7.4 functions well for most plant material.

Paraformaldehyde is a sufficient fixative for immunolight microscopy but one has to be aware that material can not be used for electron microscopy. Paraformaldehyde fixation alone is reversible so it is not advisable to store samples after fixation. If one has to store them for few days, 1% paraformaldehyde can be added to the storage buffer.

When dissecting the tissue pieces from, for example leaves, one has to be aware of the size and orientation one is interested in studying. Look at the embedding molds you are going to use with resin embedding, and estimate the suitable size and orientation of the tissue. Cut the sample pieces in a drop of fixative, and collect in glass vials, which are easy to use throughout fixation and further steps.

Fixative should be as fresh as possible. Routinely, stock solutions of 10% paraformaldehyde and 25% glutaraldehyde can be frozen in 10 ml and 1 ml aliquots, respectively. Prior to using, melt paraformaldehyde in water bath (60°C) and mix vigorously. Volume of the fixative should be clearly more than the volume of the tissue pieces (5-10 times). If the sample pieces do not sink in the fixative, use very careful vacuum treatment. After this, samples are incubated in the fixative in slow motion to

ensure even access of fixative to all sample pieces. With small tissue pieces, for example pieces of roots and leaves, fixation time of 2-4 hours at room temperature is enough, whereas overnight fixation at 4°C may be considered with seeds. After fixation, the extra fixative is washed off with at least three changes of buffer.

2.2 Agarose embedding

Encapsulating cells from cell suspension in agar or agarose makes it possible to handle loose material for microscopy [15, 16]. Agarose embedding can be even useful with tissues for three reasons: achieving right orientation of samples, centralizing the sample in the cutting surface of the resin block and in case of fragile, tiny samples, protecting the sample from drying and wounding by tweezers. For example, studying very young tissue material or very flat tissues may gain from agarose embedding prior to dehydration and resin embedding. Agarose does not influence immunolabeling as it does not penetrate tissues. This step can unfortunately not be performed before fixation as agarose is water-soluble. According to our own comparisons, 3% agarose is easiest to handle. The agarose is kept liquid in a water bath. Cast a thin layer of agarose in a petri dish or teflon mold, add tissue pieces and cast another layer of agarose. Let solidify on ice, and with a scalpel, form agarose blocks containing samples in suitable orientation. Note that the agarose blocks can not be larger than the molds to be used in resin embedding. Proceed directly to dehydration.

2.3 Dehydration and resin infiltration

An increasing series of ethanol is the most common dehydration method. If the samples, such as oat seeds, contain high amount of lipids acetone may be needed. A series of 10, 30, 50, 70, 90, 95% and three times absolute ethanol has appeared to function well with plant material. In case the fixed samples are enclosed in agarose, the blocks may appear rather soft at the first step of dehydration. London Resin White tolerates some water, and it is possible to start the embedding from 70% ethanol. Our own experience, however, has shown that it is always best to proceed to 100% ethanol in order to get proper sectioning quality [16].

Low-viscosity medium LR White suits well for plant material as it penetrates tissues easily. Medium grade LR White is suitable for most tissues, though hard grade may be better with seeds. However, LR White may also penetrate the vials, such as old porous glass beakers or polystyrene vials. Most often we have used new 10-20 ml glass vials which can be thrown away after use. Vials are covered with polyethylene locks; parafilm would be diluted by LR White vapour. Infiltration of dehydrated samples is performed with increasing concentrations of LR White mixed with ethanol, for example 30%, 60% and 100% LR White one hour each, in slow motion in the hood, followed by LR White overnight and one more change following morning.

2.4 Embedding, polymerization and sectioning

For flat embedding special Teflon molds (with 16 wells in one row) can be bought (Ted Pella, CA); LR White would penetrate the more common rubber molds. Teflon mold is supported by a metal mold which keeps the Teflon one straight when in the oven. Add some LR White in the wells, place the specimens in the short ends of the wells, and carefully fill with LR White. Check the orientation of the samples, possibly under stereo microscope. It is advisable to leave the outermost wells without specimen, just filling them with LR White.

Oxygen prevents polymerization of LR White. This is why the mold has to be covered with a special film before polymerization (Aclar film, Ted Pella, CA). Some patience is needed when placing the film as air bubbles very easily get trapped under the film. Start placing the film from one end of the mold, and lower the film gradually towards the other end. If air bubbles tend to form, add some drops LR White. Check that LR White is not running between the Teflon and metal molds. Transfer the mold carefully to an oven preheated to 58°C; the oven should be in the hood. Take the mold out next day, after 20-24

hours; check that solid blocks have been formed. Before sectioning, the blocks are left in the hood for few days. Post-polymerization at 40°C for a couple of days can be done if the blocks seem soft. Sectioning quality of the blocks gets better within time. According to our experience, polymerization by heath usually functions well. There is, however, a risk for decreasing antigenicity. Cold polymerization by UV light is possible with LR White as well.

Alternatively to Teflon molds, embedding can be done in upright position in gelatine capsules. The capsules have to be dried for at least 4 hours at 58-60°C prior to embedding; otherwise LR White leaks out. Microtiter plates are handy as racks. Instead of Aclar film, the capsules are covered with their own counterpart.

The blocks are not automatically marked when using Teflon molds or gelatine capsules. Care has to be taken not to mix the samples. For marking, small pieces of paper can be added in the wells or placed like a ring inside gelatine capsules. Thin lead pencil is best for marking; when using printed labels, we found traces of ink on the samples.

Semithin sections of 1-2 µm are cut for immunolight microscopy. SuperFrost Plus (Menzel-Gläser, Braunschweig, Germany) slides ensure good adhesion. It is practical to have two groups of 2-4 sections on a slide; one for the antibody and one for the control. Prior to immunolabelling, waxy lines can be drawn round groups of sections to keep the incubations separate. For ultrathin sectioning, 200 mesh nickel grids are the usual choice. Collect few sections on a grid. Section thickness may be varied.

3. Immunolocalization

Immunolocalization method has the following steps: blocking, incubation with primary antibodies, wash, incubation with secondary antibody conjugate, wash and putative visualization step, wash, putative counterstaining and mounting. Detailed descriptions of these steps can be found in several references [12, 13, 14]. In the following we discuss the main prerequisites of immunolocalization.

3.1 Primary antibodies

The ability of the primary antibody to recognize its antigen is the key of immunolocalization method [12, 14, 17]. It is important to know 1) against what antigen the antibody was raised; 2) is it a monoclonal or polyclonal antibody; 3) if it is polyclonal antibody, in what animal it was produced in; is it a crude antiserum, isolated IgG fraction or affinity-purified antibody; 4) is there pre-immune serum available for polyclonal antiserum; 5) what titers have been used previously, for example in western blotting; 6) is the concentration known?

Part of the epitopes is always covered by fixation and tissue processing. A monoclonal antibody is highly specific as it recognizes only one type of epitope in the antigen. This may sometimes be a problem if the particular epitopes were masked by fixation. Polyclonal antiserum, which consists of a mixture of antibodies against different epitopes in the antigen, can be advantage in immunomicroscopy, though the risk for non-specific binding is higher. If background labelling is a problem, isolation of IgG fraction of the antiserum can be done. To achieve even more specific antibody, affinity purification may be tried in case there is some pure antigen available. However, if the affinity between the antigen and antibody is extremely strong, it may be almost impossible to elute the bound antibodies from the column. In case of a glycoprotein, there is a danger that the antiserum contains antibodies against the glycosyl residues thus adding the non-specific binding considerably (Fig. 1). These can be separated from the antiserum [18]. However, there are highly specific antibodies for studying polysaccharides for example in plant cell walls [19].

Titer for the primary antibody has to be optimized for immunomicroscopy. As a rough rule, one can estimate that if dilution of 1:1000 functions well in western, then 1:100 is suitable for light microscopy, and 1:10 for TEM. There are, of course, exceptions. Usually the specificity of the primary antibody has been tested with some other method, such as immunoblotting (western), dot blotting, immunotissue printing, or ELISA. It is advisable to check the specificity against both a pure antigen and tissue extracts. Western is commonly used to test antibodies; however, different conformation of antigen in western and

on tissue sections may sometimes give different results. Immunotissue prints, if possible to produce of the material, may be used instead or together with western blots.

For immunomicroscopy, a series of primary antibody dilutions are tested *together* with preimmune (or nonimmune) serum diluted to the same concetration. One should try to find the antibody dilution which gives clear labelling with minimum background. Long incubation times, i.e. overnight at 4°C, are preferred for resin sections. Antibody is usually diluted with 1% BSA in PBS thus mimicking the natural environment of antibodies.

Immunolight microscopy is always performed before immunoelectron microscopy.

3.2 Controls and interpretation of results

Different controls are needed to verify the results [12, 14]. One is looking if there are differences between the samples and between the different treatments. As mentioned above, a positive and negative tissue control would be a good pair for comparison, for example an infected and non-infected tissue. Together with primary antibody incubation one performs incubation where the primary antibody is substituted with preferably pre-immune serum, i.e. serum from the same animal where the primary antibody was produced taken before immunization. If this is not available, commercial non-immune serum from the same species can be used. One can also perform a control using dilution buffer instead of the primary antiserum in order to see if the secondary antibody conjugate creates background staining. An alternative is to use primary antiserum blocked with its antigen as a control. It is important to consider carefully before immunolocalization what controls are needed and why. In case there is no positive signal at all, one could have use of a known positive primary antibody in order to see if the method functions. Some further controls are mentioned when discussing visualization methods.

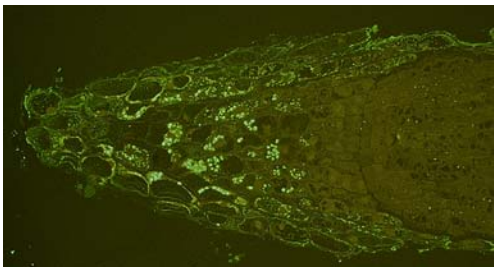

Fig. 1 False positive labelling seen as immunofluorescence on a semithin LR White section of barley root tip. The polyclonal antiserum contained anti-glycan antibodies which reacted with root cap cells.

3.3 Choice of visualization method

It is convenient to use immunogold labelling as visualization method as it suits for both light and electron microscopy. At electron microscope level, 10 and 15 nm gold particles are routinely used; they are relatively easy to find at the sections (Fig 2.). If using small gold particles at TEM, they can easily be enhanced with the same silver kit as for LM. If one is planning to perform quantification of gold label, protein A coupled to gold may be preferred as it binds only to one epitope [10]. Secondary IgG may bind either to one or two epitopes. However, protein A may bind to some special glycoproteins at plant tissues [20]. This can be checked by omitting the primary antibody. Protein A easily recognizes antibodies from rabbit whereas reaction is weaker with chicken or mouse antibodies.

For light microscopy, small gold particles (1-5 nm) are used and enhanced with silver. Silver reaction is both light and temperature sensitive. Silver precipitate is formed around the gold particles; this may cause problems in finding suitable focus for photographing, especially if the tissue of interest has secondary cell walls. Silver precipitate may also cover cells making it sometimes difficult to see where exactly the label is located. Possibly one could try checking the sections at reflection mode in confocal microscope. Counterstaining, if needed, is performed before mounting the sections.

Alternatively for immunogold silver one can test secondary antibodies conjugated to enzymes, such as alkaline phosphatase or horse radish peroxidase. Colour reaction coupled to enzyme substrate reveals site of labelling. Putative endogenous enzyme activities have to be checked by doing the colour reaction without the secondary conjugate. Immunofluorescence is an excellent alternative for visualization as one does not need to perform separate colour or silver reaction. It functions well with semithin resin sections [21]. It is best to check some sections without secondary conjugate in order to see the level of autofluorescence at the tissue. One can consider choosing fluorochrome of another colour than the autofluorescence, if it is strong. There are even other visualization methods such as avidin-biotin or TSA amplification; one should remember, though, that more sensitive methods also create background more easily.

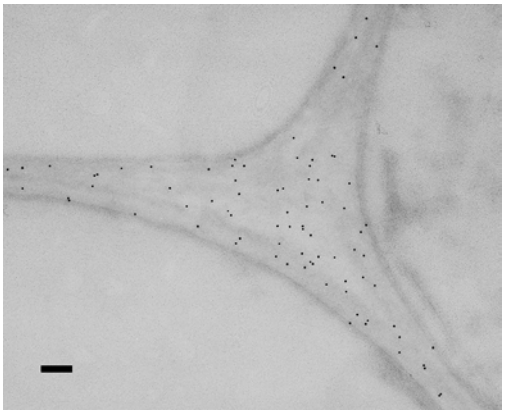

Fig. 2 An LR White section from infected barley root cortex showing immunogold labelling of PR-3 pathogenesis-related protein. Gold particles of 10 nm are scattered in the cell wall. Scale bar 200 nm.

3.4 Trouble shooting

Too strong signal is easier to handle than no signal at all, as one can start to optimize the positive labelling [12, 14]. Diluting primary antibody and the control serum more is the first thing to try. If the sections have dried at some point during the series from blocking to the last wash after secondary antibody conjugate, high background is likely. One can change the reagents to new ones, for example a silver enhancement kit easily gives background if too old. Stronger blocking, adding 0.1% Tween 20 to the washing buffer and even to the antibody dilutions can be tested. One should work systematically and change only one factor at the time. Checking with higher magnification in light microscope can reveal differences not seen with low magnification.

Lack of positive labelling is even more challenging. This is why one should optimize the method with start material that is known to contain the relatively abundant amount of antigen. Otherwise one can speculate that the lack of label is due to no antigen in the tissue. First thing to test is stronger primary antibody dilution; the control serum has to be diluted to the same concentration. A longer incubation time and higher temperature up to 40°C can be tested. Check the secondary conjugate, for example gold particles tend to loosen from the conjugate with time. Dilution series with secondary antibody conjugate can be done.

Fading of signals in light and fluorescence microscopy is common. One can retard this by keeping the samples at 4°C and using anti-fading agent in fluorescence [13]. In any case, it is best to check and document the samples within a couple of days. On the contrary, gold labelling on grids do not disappear. When taking photographs, try to adjust the digital image as near the image you see in the microscope as possible, and use same magnifications with different samples.

4. Concluding remarks

Here we have presented some common advice to beginners at immunomicroscopy based on our own experience. It is good to do a pilot study as optimizing is always needed with new material and primary antibody combination. It is also important to be aware of laboratory safety aspects, as many chemicals used in microscopy laboratories are harmful or toxic. With careful planning there is a good chance to succeed with immunomicroscopy studies.

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