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IMMUNOGOLD LOCALIZATION OF INTRA- AND EXTRA-CELLULAR PROTEINS AND POLYSACCHARIDES OF PLANT CELLS

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Abstract

This paper illustrates post-embedding immunogold labelling of protein and polysaccharide molecules of plant cells. For EM studies, one is restricted (for most plant cells) to the post-embedding approach because the surrounding cell wall prevents access of antibodies (and secondary gold-tagged markers) to internal sites. The large size of many plant cells also does not lend itself to diffusional entry of antibodies. The molecules localized include seed storage proteins that are large and present in major quantities, a smaller less abundant, water soluble albumin, an oxygen-binding protein, components of the photosynthetic electron transport chain, and complex sugars from the cell wall. A range of preparative procedures and embedding plastics are used.

Key Words: Immunogold, plants, seed storage proteins, root nodules, photosynthesis, cell walls.

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Introduction

The locations of specific molecules can be revealed with suitably tagged ligands such as antibodies using two approaches: pre- and post-embedding labelling. In pre-embedding labelling, antibodies are applied before embedding the tissue or cells in plastic and sectioning for microscopy; in post-embedding, antibodies are applied after sectioning. Elegant studies have been done with animal tissues using both approaches (Willingham et al. 1981; Langanger et al. 1984; Roth 1986).

In pre-embedding labelling, antibodies are required to diffuse into the tissue, a process that may require appreciable time because of the size of the antibody molecules. Even longer times are necessary if the antibodies are tagged with a marker such as colloidal gold. Post-embedding labelling, on the other hand, exposes target molecules on the surface of the section for "easy" access by the antibody (molecules below the surface of the section will not be labelled; Bendayan 1984). However, post-embedding labelling suffers, at least potentially, from the chemical treatments necessary to embed and section the tissue, all of which may cause loss of recognition between the antibody and its complementary antigen (Craig and Goodchild 1982).

With few exceptions, plant cells are large (typically 50-100 μm in diameter) compared to animal cells. This imposes constraints on the entry by diffusion of antibodies. More important, however, plant cells are enclosed by a cell wall consisting of a network of cellulosic fibrils impregnated with a matrix of proteins, polysaccharides, lipids and other substances. The pore size of the cell wall has been determined to be <5 nm (Hoggart and Clarke 1984). Antibodies, having dimensions of at least 5 nm, should not diffuse into plant cells through the cell wall.

Immunolocalization by electron microscopy of antigens beneath the surface of plant tissues is thus restricted to the broad post-embedding protocol described above. Published tissue preparation protocols vary, but most studies have used fixation with aldehydes. Perhaps of more interest is the wide range of embedding plastics that have been used with success, including Spurr's epoxy, GMA, Lowicryl K4M and the methacrylates L R White and L R Gold.

Immunofluorescence localization studies in plant cells have been discussed (Knox et al. 1980; Jeffree et al. 1982), but as yet no general consideration of immunolocalization studies in plant tissues by electron microscopy is available. In this report, localization of a range of intra- and extra-cellular antigens in plant tissues will be described, and some general comments and observations, based on several years of experience in the field will be offered.

Materials and Methods

Tissue preparation

A variety of different plant tissues and organelles were studied, including cotyledons of developing pea seeds, nitrogen-fixing root nodules of soybeans, root tips of onion seedlings, and chloroplasts isolated from spinach leaves. In all cases, the tissue was fixed in glutaraldehyde (GA, 0.1-3%). A mixture of freshly dissolved paraformaldehyde (PFA) and GA (usually 2% and 0.1% respectively) has also been used successfully. Fixation was usually done on ice, although for many of the samples, this did not result in improved labelling over that seen with fixation at 22°C. Many samples were post-fixed in 1-2% OsO_4.

Dehydration was through a graded series of ethanol, followed by infiltration and embedding in either Spurr's resin, Lowicryl K4M or L R White by standard protocols (for example, see Craig and Miller 1984). For embedding in L R Gold, tissue was dehydrated through methanol. Polymerisation was initiated with, respectively, heat (70°C), UV light, heat (50°C) and white light as per manufacturers' recommendations.

Antibodies

The following antibodies were used:

1) Affinity purified antibodies to the Mr 50,000 polypeptide complex of the globulin storage protein vicilin of pea cotyledons, described in detail by Craig and Goodchild (1984).

2) An ammonium sulfate cut of antisera raised against a sulfur-rich albumin (PA1) of Mr 6,000 from pea seeds. See Higgins et al. (1986) for further characterisation of this preparation.

3) Immunoglobulin preparations of antibodies raised against either rhamnogalacturonan (RG-1) or xyloglucan, components of plant cell walls (Moore et al. 1986).

4) An IgG fraction (isolated from a protein-A affinity column by Dr D.J. Goodchild) of antibodies raised against an oxygen-binding protein, leghaemoglobin, from soybean root nodules.

5) Antibodies raised against the main apoprotein of the chlorophyll a/b-light harvesting complex (chl a/b-LHC) of photosystem II (PSII) and against the photosystem I reaction center complex CPI, both isolated from spinach chloroplasts (Dunahay and Staehelin 1985,1986).

Immunocytochemistry

Sections were collected on parlodion- or formvar-carbon coated nickel grids. Antibody labelling was performed in the following steps:

1) "block" with bovine serum albumin (10 mg/ml) to saturate non-specific sites of protein adsorption.

2) primary antibody - preferably at a protein concentration of 10-50 µg/ml, but as low as possible to eliminate non-specific binding, and not to reduce specific binding.

3) buffer wash - 5-10 secs. from a squeeze bottle.

4) protein A-gold or goat anti rabbit-gold (GARG, Janssen Pharmaceutica, Beerse, Belgium). Protein A-gold was prepared according to Frens (1973), or Slot and Geuze (1985).

5) buffer wash, followed by distilled water rinse.

6) post stain with lead and/or uranyl salts.

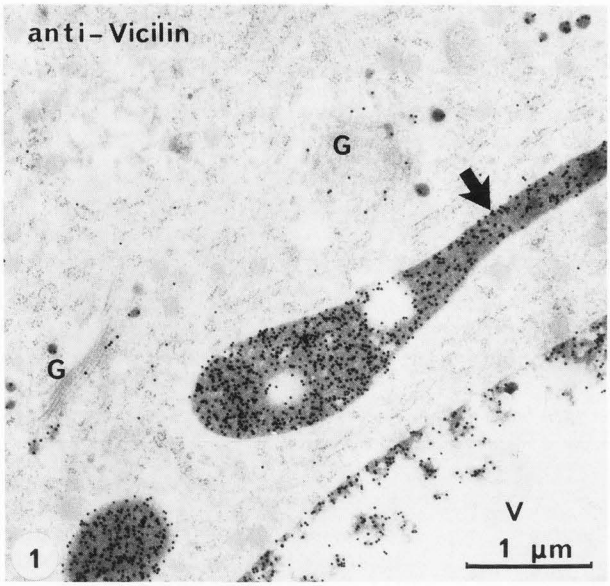
Antibodies and gold were carried in 25 mM phosphate buffer containing 500mM NaCl and 0.1% Tween 20 (see Craig and Goodchild 1982). This buffer mixture was also used for washing the sections. Sections of tissue that had been post-fixed with OsO_4 were treated with $NaIO_4$ and HCl before antibody labelling, as described by Craig and Goodchild (1984). Incubation steps were for 10 min at 22°C unless stated otherwise.

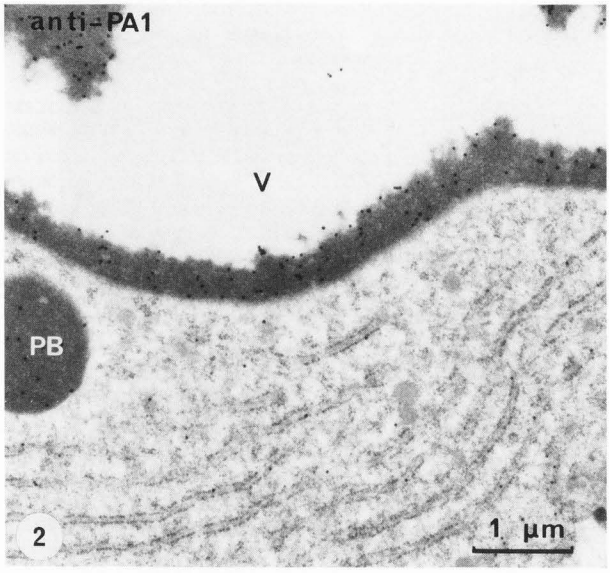
Results

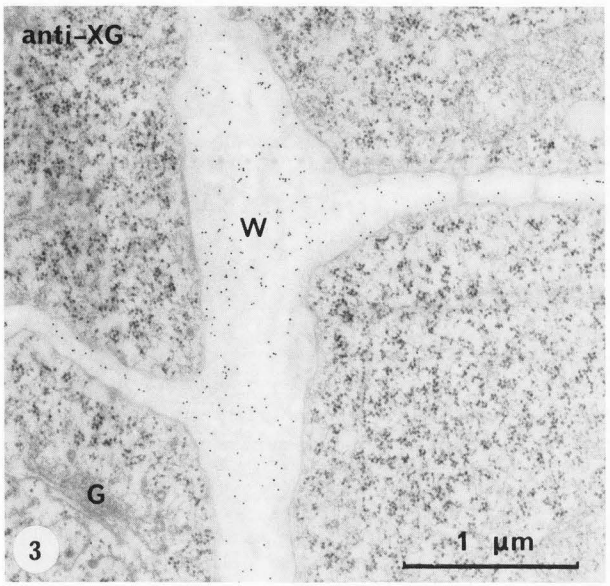
Representative micrographs of labelling obtained with the antibodies described are shown in Figures 1-6. A number of general points can be drawn from these micrographs. First, a variety of antigens can be localized in plant tissues fixed with standard fixatives (GA or GA/PFA) that are capable of very good fine structural preservation. Second, a variety of resins are readily applicable to immunolocalization studies with plants. Also, both protein and polysaccharide antigens can be localized with these techniques.

In tissue fixed with GA and OsO_4 and embedded in L R White, storage proteins were localized in cotyledons of developing pea seeds (Figs. 1 and 2). Vicilin (Fig. 1), which accounts for up to 40% of the total seed protein, was associated with rough endoplasmic reticulum (ER), its site of synthesis, with Golgi apparatus, where glycosylation of at least some of the peptides occurs, and was present in vacuoles and protein bodies. The latter are the sites in which protein is stored until it is required for seedling growth following germination. A second storage protein, a water soluble, sulfur-rich albumin of Mr 6,000 (PA1) that is a much less abundant storage protein than vicilin, was also detected within the rough ER and in vacuoles and protein bodies (Fig. 2). Anti-PA1 also bound to some, but not all Golgi apparatus; perhaps there was insufficient antigen present to be detected (Craig unpublished). PA1 accounts for ~1% of the seed protein, compared with 40% for vicilin, and this is reflected in the density of bound gold.

Plant cells secrete a variety of molecules, the most prominent being the constituents of the wall that surrounds each cell. As already mentioned, the cell wall consists of cellulose microfibrils impregnated with many other







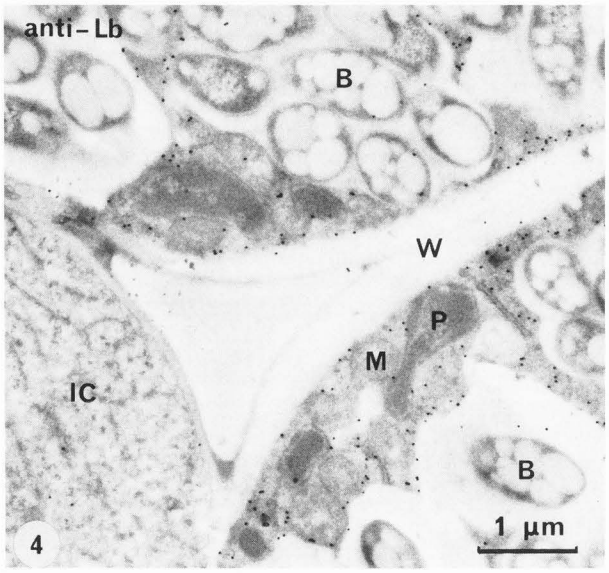


Fig. 1. Localization of the storage protein vicilin in developing cotyledon of pea seed. Antigen is present in the trans cisternae of Golgi apparatus (G), in vacuolar (V) deposits of protein, and in large protein-filled cisternae (arrow). Although not shown, vicilin was also associated with the ER. Fixed with GA, post-fixed with OsO_4, and embedded in L R White.

Fig. 2. Localization of a sulfur-rich albumin storage protein (PA1) in developing pea cotyledons. PA1 is associated with the ER and is stored within vacuoles (V) and protein bodies (PB). Compared with vicilin (Fig. 1), less gold is bound after reaction with anti-PA1, reflecting the approximately 40-fold difference in content of the two proteins. Preparation as in Fig. 1.

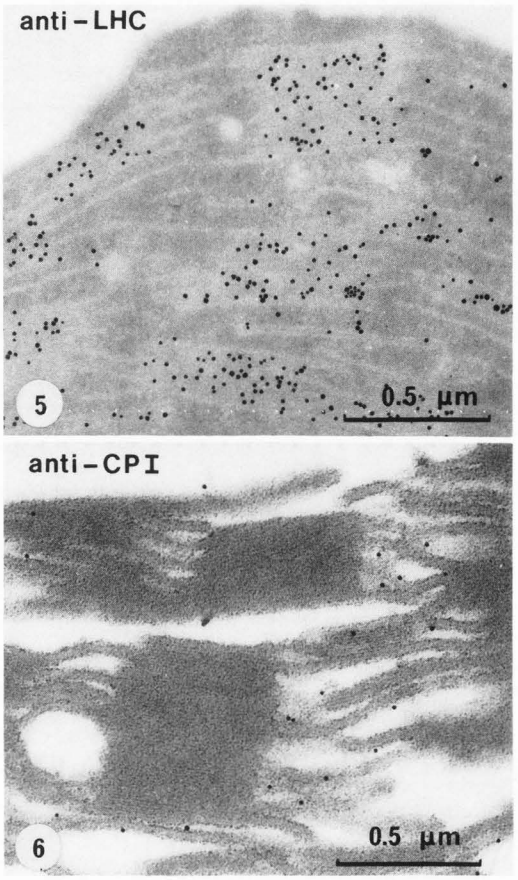
Fig. 3. Localization of xyloglucan, a complex sugar, throughout cell walls (W) of young onion root tip cells. G: Golgi apparatus. Preparation as in Fig. 1.

Fig. 4. Localization of leghaemoglobin, an oxygen-binding protein in cells of a root nodule from soybean. Antigen is restricted to the host cell cytoplasm, the bacteroids (B) which contain the symbiotic bacteria, having no detectable leghaemoglobin. Plastids (P) and mitochondria (M) are also unlabelled. Not all cells in the nodule are invaded by the bacteria; uninfected cells, the interstitial cells (IC), have no detectable leghaemoglobin and thus provide an internal control in the preparation. W: cell wall. Fixed with GA and OsO_4 and embedded in L R White.

components, including proteins, lipids, ions, and complex sugars thought to be involved in "glueing" the cellulose together. Antibodies were used to investigate the distribution of two such sugars, RG-1 and xyloglucan (Moore et al. 1986), within cell walls. In Figure 3, xyloglucan is shown to occur throughout the wall in growing root tips of onion. On the other hand, RG-1 was most common in the middle lamella-primary wall region (not shown).

On the roots of leguminous plants, symbiosis with certain bacteria results in the formation of multicellular nodules in which the bacteria invade the cortical cells of the root. In the resultant symbiosis, atmospheric nitrogen is converted to organic compounds that are used by the host plant for growth. For nitrogen fixation, the partial pressure of O_2 around the bacteria must be rigorously controlled, a process that is regulated by the oxygen-binding protein leghaemoglobin (Lb). Anti-Lb binds to the host cell cytoplasm external to the membrane that encloses each group of bacteria, the so-called peribacteroid membrane (Fig. 4). No Lb was detected within the bacteroids (B), a result also reported by Robertson et al. (1984), who used tissue embedded in Lowicryl K4M. Not all cells of the nodule are invaded by the bacteria. Anti-Lb did not bind to uninfected cells, the interstitial cells (IC, Fig. 4), which thus provide an excellent internal control within each section for both the method- and immuno-specificity of the labelling.

Localization of protein components within complex cellular organelles such as chloroplasts presents additional problems. Chloroplasts are bounded by a double membrane envelope which would prevent the entry of antibodies during pre-embedding labelling, even if the organelles were isolated from the plant cells. In addition, the components of the photosynthetic apparatus of chloroplasts are localized within a complex set of internal membranes. These membranes, called thylakoids, consist of an intricate system of interconnected flattened sacs which are differentiated into tightly appressed "stacked" regions (the grana) and unappressed stromal membrane regions. The tight appression of the grana membranes would also prevent antibody entry and identification of proteins specific to stacked regions. In the past several years, a large body of biochemical and structural data has indicated that the components of the photosynthetic electron transport chain are distributed non-randomly between the appressed and non-appressed membrane regions (Dunahay and Staehelin 1986). The post-embedding labelling technique has proven to be an excellent method to directly demonstrate this lateral heterogeneity within thylakoid membranes. Two examples are seen in Figures 5 and 6; in these experiments antibodies were used to localize the chl a/b-LHC and CP1 within intact chloroplasts and isolated thylakoid membranes respectively. In concurrence with the fractionation studies, the chl a/b-LHC was found almost exclusively in the grana regions (Fig. 5), while CP1, the PS1 reaction center, is localized primarily in unstacked stromal membranes (Fig. 6). The post-embedding labelling technique has also been successfully employed to localize several other components of thylakoid membranes, including the polypeptides involved in oxygen evolution in PSII, the cytochrome b6/f complex, and the CF1-CF0 ATP synthase complex (Allred and Staehelin 1985; Goodchild et al. 1985; Vallon et al. 1985).



Figs. 5 and 6. Immunogold localization of the chl a/b-LHC in intact spinach chloroplasts (Fig. 5) and of the photosystem 1 reaction center CP1 in isolated thylakoids (Fig. 6). Both samples were fixed in GA and embedded in Lowicryl K4M. The thylakoid sample (Fig. 6) was also stained en bloc with hafnium chloride to improve the contrast of the membranes within the grana stacks.

Discussion

In cells and tissues of plants, the use of antibodies and colloidal gold markers to localize and visualise intracellular sites presents a set of problems that are not encountered when working with animal cells. Because the surrounding cell wall is impermeable to both antibodies and gold-labelled secondary probes such as protein A, antibodies or avidin, one is largely restricted to labelling sections of embedded tissue, the post-embedding approach. The size of most plant cells (50-100 µm) would pose a further problem if one could gain access through the cell wall, necessitating longer times for penetration by

diffusion of the antibodies. Attempts by one of us (SC) to improve/achieve antibody penetration by partially extracting the cytoplasm and membranes with detergents incorporated into the fixative, met with negligible success. One case where pre-embedding labelling has been successful in plants is in the giant alga Chara. Here, single cells may be up to 1 mm in diameter and 100 mm in length. The ends of the cell can be excised and the large central vacuole removed, which allows antibody solutions to be perfused through the cell (Williamson et al. 1986).

A corollary of the large size of plant cells is that it takes significantly more time for fixation than is required with cultured animal cells. In many instances, the large size of plant cells is related to the presence within them of a large, central vacuole. The vacuole is extremely difficult to stabilize, and when ruptured, its lytic contents can destroy cell ultrastructure. This problem is accentuated by the common desire to use freshly prepared paraformaldehyde as the fixative. PFA is osmotically very active (Goodchild and Craig 1982) and vacuoles can rarely be stabilized with this fixative; they generally lyse in fixatives of high osmolality, resulting in poor ultrastructure.

Plant cell walls are rich in charged sugar residues and thus are able to adsorb by non-specific means many antibodies and gold-labelled markers. Some cell walls are impregnated with phenolic materials (these are commonly described loosely as lignified walls) and these have even greater tendencies to bind antibodies non-specifically. Thus, particular care must be exercised in establishing the specificity of labelling when dealing with cell wall-bound antigens. Many cell walls are autofluorescent. This, in concert with GA-induced autofluorescence can make immunofluorescence a very difficult task with plants (Craig et al. 1979). Recent developments in enhancing gold-labelled sites with silver (Danscher and Norgaard 1983; VandenBosch 1986) are particularly exciting and hold great promise to circumvent the problems associated with autofluorescence in plant cells.

The choice of resin for embedding can be important. Different resins may result in different patterns of antigen detection, depending on their interaction with the antigen in question. For example, when pea cotyledon was embedded in Spurr's epoxy, the storage protein vicilin could be detected in Golgi vesicles, but not within the Golgi cisternae (Craig and Goodchild 1984). However when L R White was used for embedding, the protein was detected in both cisternae and vesicles. Also, the density of bound gold was greater than that on Spurr's embedded tissue (Craig and Miller 1984). Still greater density of bound gold was seen when either Lowicryl K4M (Craig and Goodchild 1982) or L R Gold (Craig and Miller, unpublished) was used for embedding pea cotyledon tissue. However with these latter two resins, antigen was not detected in sites over and above those detected using L R White, and because OsO_4 cannot be used with the former resins, the fine structure was not well contrasted and the preservation appeared to be inferior. In this example, the combination of antigen detection with tissue preservation and contrast obtained with GA/OsO_4 and L R White were considered preferable to a higher density of label over less-well resolved fine structure. On the other hand, α-amylase localization by electron microscopy in plant cells had proven unsuccessful until recently, when Lowicryl K4M was found to be the only resin suitable for its preservation and localization (Gubler et al. 1986).

The use of osmium fixed tissue warrants much more study. It is clear that many antigens are not destroyed by OsO_4, especially when it is used following GA fixation (Bendayan and Zollinger 1983; Craig and Goodchild 1984). Epitopes may be masked (probably sterically) from access to the Fab sites on the IgG molecule, but they may be exposed by chemical pretreatments such as sodium metaperiodate that are thought to remove osmium from the section surface. Similarly, we suggested that further epitopes may be revealed by removal of GA from the section surface with dilute HCl (Craig and Goodchild 1984). Further progress along these lines may be expected.

Monoclonal antibodies have not yet been widely used in studies of plant cells by electron microscopy. Monoclonal antibodies were used in the pre-embedding labelling of Chara already mentioned (Williamson et al. 1986). However all attempts at post-embedding labelling with these antibodies were unsuccessful (Craig, unpublished). Similarly, we used monoclonal antibodies against another pea storage protein (legumin), but with no success (Craig and Hewish, unpublished). Thus, the potential advantages of monoclonal antibodies with immunolocalization have not yet been realised with plant cells.

The observation that one can localize antigens such as the small, water soluble albumin, PA1 (Mr 6,000), and the oxygen-binding protein Lb that is known to be mobile and to undergo marked changes to its properties upon exposure to GA, provides great encouragement that further progress in the use of colloidal gold to reveal intracellular molecules in plant cells is likely. Also, the ability to produce monodisperse gold particles of a range of sizes opens the way for double labelling studies that can provide spatial information not currently available with other techniques (Craig 1986).

Summary

Plant cells and tissues present certain unique problems with regard to immunolocalization studies by transmission electron microscopy. This brief overview is drawn from studies of several different tissue systems and serves to illustrate the widespread applicability of localizing specific target molecules with antibody probes. Using antibodies and colloidal gold, protein and polysaccharide molecules have been localized in different tissues and in isolated organelles of plants. Because plant cell walls are impermeable to antibodies, the post-embedding approach must be used. Aldehyde fixation, followed in some cases by osmium, and embedding in a variety of plastics, including

epoxy and methacrylates, enabled successful antibody labelling on material with good fine structural preservation and tissue contrast. No single fixation and embedding protocol was found to be satisfactory for all of the antigens studied; post-fixation with osmium was compatible with several antigens, provided the sections were treated with sodium metaperiodate prior to antibody labelling. The molecules localized include seed storage proteins, an oxygen-binding protein, components of the photosynthetic electron transport chain, and complex sugars from the cell wall. We suggest that immunogold labelling, perhaps in concert with silver enhancement, should also be applicable to studies of the same (and additional) molecules at the light microscope level. If this suggestion is correct, the method would overcome the considerable problems associated with autofluorescence of plant tissues.

Acknowledgements

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Discussion with Reviewers

G.H. Haggis: What are the possibilities of antibody labelling cryosections of plant material following the methods introduced by Tokuyasu (J. Cell Biol. 57, 551-565, 1973)?
Authors: This approach has been used successfully with plant tissue by Baumgartner et al. (Planta 150, 419-425, 1980). Poor contrast, as tends to be the case with cyrosections, restricted the information that was obtained. Better contrast was gained by Greenwood et al. (Planta 164, 295-302, 1985) where cryosections, cut and antibody-labelled as per Tokuyasu's method, were refixed in glutaraldehyde and osmium, dehydrated and embedded (all on the grid) in epoxy or L R White resin. The material used by Greenwood et al., developing legume seed tissue, was very similar to that used in Figures 1 and 2; no additional information was gained through the use of cryosections.

G.H. Haggis: In a paper on immunogold labelling for thin-section electron microscopy, it is not allowable and irrelevant to introduce remarks about immunofluorescence.
Authors: On-grid immunogold labelling has been used successfully to localize a range of target molecules in plant tissues. To repeat these observations in the light microscope by immunofluorescence would be difficult, or perhaps not possible, because of tissue autofluorescence. Figure 4 in Craig et al. (1979) demonstrates the problem of autofluorescence, and similar observations were made for the cell wall polysaccharides described in Figure 3 of this paper by one of us (P J M). Since immunogold labelling can be used for light microscope immunocytochemistry, we think that it is appropriate to mention this fact briefly.

C. Sauter: Many technical details, such as tissue preparation protocols, antibody concentrations and control reactions, are lacking, and the biological meaning of the results is not discussed.
Authors: This paper is presented as an overview of published work to show: a) the diversity of approaches that can be successfully used for immunogold localization studies with plant tissues, and b) the range of molecules that can be detected with these approaches. Apart from the anti-leghaemoglobin, for each example illustrated, full details of tissue preparation, antibody preparation, and control reactions used to establish the specificity of both the method and antisera, are given in the quoted reference(s). It did not seem appropriate to reproduce them here.

To establish the biological meaning of the results presented would require pages of additional text and, again, the biological implications of the observations have been discussed in the cited references. The significance of leghaemoglobin localization in cells of legume root nodules has been addressed by Robertson et al. (1984). The "biological implication" of this paper is that a range of molecules (protein, polysaccharide), large or small, from extra- and intra-cellular sites, can be localized with on-grid immunogold labelling.