

Immunofluorescence microscopy and immunogold EM for investigating fungal infection of plants

Christopher R Thornton & Nicholas J Talbot

School of Biosciences, University of Exeter, Geoffrey Pope Building, Stocker Road, Exeter EX4 4QD, UK. Correspondence should be addressed to C.R.T. (c.r.thornton@ex.ac.uk) or N.J.T. (n.j.talbot@ex.ac.uk)

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Fungi are a diverse group of eukaryotic organisms whose activities are intricately linked to the lives of human beings. Their involvement in plant productivity, as agents of human diseases, as sources of medicines and enzymes and as model experimental organisms has necessitated the development of sensitive and specific techniques for tracking the organisms and their protein products. Techniques employing highly specific monoclonal antibodies have allowed the visualization of fungi in their natural environments and have facilitated the study of their antigens at the subcellular level. Here, we describe three such techniques, immunofluorescence (IF), immuno-enzymatic staining (IES) and immunoelectron microscopy (IEM), that have found widespread applicability in studies of fungal biology, and which can also be adapted for use in the study of other eukaryotic organisms. Results from the IF and IES procedures can be obtained within 4–5 h. Sample preparation for IEM takes approximately 4 days. Gold labeling and visualization of samples can be completed within 4 h.

INTRODUCTION

These protocols describe the use of monoclonal antibody (mAb)-based assays for the immunolocalization of fungal antigens. The protocols can be used for studying all aspects of the biology of fungi that require visualization of antigens. These may include imaging of the whole organism in its natural environment using mAbs targeted against cell-surface antigens¹ or tracking the spatio-temporal expression of an individual protein during fungal morphogenesis^{2,3}. The protocols can equally be applied to the study of other eukaryotic organisms that require localization of antibody–antigen interactions, provided appropriate antibodies are available.

Fungi are a diverse group of eukaryotic microorganisms that constitute a kingdom different from plants and animals. Their relatively short lifecycle, genetic variability and ease of culture in the laboratory lend themselves well to genetic analysis, and fungi such as the unicellular yeast *Saccharomyces cerevisiae* serve as paradigms for understanding many fundamental processes in eukaryotes such as the cell cycle. The importance of fungi as model organisms is reflected in the release of the yeast genome sequence in 1996 (ref. 4), the first eukaryotic genome to be analyzed completely.

Since then, the genomes of a large number of fungi have been sequenced, including human and plant pathogens^{5,6} and fungi of industrial importance⁷, and this has opened the door to systematic screens of gene function in these organisms. The inactivation of genes by homologous recombination has allowed us to determine which genes are essential in the life cycle of these fungi, and the widespread use of green fluorescent protein⁸ and related fluorescent proteins as reporter molecules has greatly facilitated the study of

protein expression in the fungal cell⁹ without the need for chemical fixation and staining. Nevertheless, it is important to note that such studies are limited to a handful of organisms that are genetically tractable and where transformation systems are readily available. Consequently, there is still widespread demand for substitute techniques such as the antibody-based assays that provide alternative means to study protein distribution and dynamics in less well-characterized fungi. Many antigens (proteins and glycoproteins) involved in the key processes of adhesion, cell wall penetration and tissue colonization, which are themselves the subject of genetic manipulations¹⁰, are extracellular or cell wall-bound and their spatio-temporal localization can provide valuable information as to their function in the biology of the organism.

The protocols described here provide simple methods for the immunolocalization of fungal antigens using IF, IES and IEM procedures. The protocols have been developed specifically for the visualization of extracellular antigens or antigens bound to the surface of cells. They are not suitable for the identification of intracellular antigens where alternative strategies must be adopted¹¹. The protocols can be employed as templates for localizing antigens from any fungus, provided suitable antibodies and appropriate microscopes are available. The protocols described here employ mouse mAb, the production of which is described elsewhere¹². However, they can be adapted for use with polyclonal antisera, thereby extending the range of immunolocalization studies that can be carried out¹³.

MATERIALS

REAGENTS

- mAb tissue culture supernatant (mAb TCS)
- Tissue culture medium (TCM) control
- Goat anti-mouse polyvalent (IgG, IgA, IgM) fluorescein isothiocyanate (FITC) conjugate (Sigma)
- Goat anti-mouse polyvalent (IgG, IgA, IgM) alkaline phosphatase (AP) conjugate (Sigma)

- Goat anti-mouse IgG+IgM 20, 10 or 5 nm colloidal gold conjugates (British Biocell International)
- Casein (BDH)
- Sodium azide solution (0.05%; wt/vol) **! CAUTION** Sodium azide is a highly toxic chemical
- Phosphate-buffered saline (PBS) buffer: 137 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.4

- PSB with Tween 20 (PBST) buffer: PBS buffer containing 0.005% (vol/vol) polyoxyethylene(20)sorbitan monolaurate (Sigma)
- PSB-glycerol mounting solution (Sigma)
- Naphthol-AS-phosphate (Sigma) ! CAUTION Naphthol-AS-phosphate is an irritant
- *N,N*-dimethylformamide (Sigma) ! CAUTION *N,N*-dimethylformamide is a toxic chemical
- Fast Blue BB diazo salt (Sigma) ! CAUTION Fast Blue BB is an irritant
- Paraformaldehyde EM (TAAB Laboratories) ! CAUTION Paraformaldehyde is a harmful chemical
- Glutaraldehyde (Sigma) ! CAUTION Glutaraldehyde is a toxic chemical.
- LR White resin (Agar Scientific Ltd)
- Gelatine capsules (Agar Scientific Ltd)
- Piloform (Agar Scientific Ltd)
- Bovine serum albumin (BSA) (Sigma)
- Tris (Melford)
- Parafilm

EQUIPMENT

- Epifluorescence UV microscope fitted with 495 nm (excitation) and 518 nm (emission) filters for FITC. For other fluorochrome conjugates, the appropriate excitation and emission spectra should be used—for example, Texas Red (Calbiochem) (peak emission 615 nm), Alexa 430 (Molecular Probes) (peak emission 541 nm) and Cascade Blue (Molecular Probes) (peak emission 421 nm)
- Portable UV lamp (model B7960, wavelength 360 nm) for LR White resin polymerization (Agar Scientific Ltd)
- Ultramicrotome fitted with a glass or diamond knife
- Transmission electron microscope

PROCEDURE

1 | Grow the fungus on an appropriate surface, glass slide¹³, leaf or root tissue. In the example of IF shown in **Figure 1**, spores of the foliar rice pathogen *Magnaporthe grisea* were inoculated onto the surface of onion epidermis. In the example of IES shown in **Figure 2**, biocontrol strain of the soil-borne fungus *Trichoderma hamatum* was allowed to colonize the roots of lettuce plants grown in soil. These were carefully removed from soil and processed immediately. Proceed with IF (option A), IES (option B) or embedding and gold labeling (option C). IES staining is a good substitute for IF where autofluorescence from plant material interferes with fluorescence from a fluorochrome conjugate. This is typically the case when using the excitation and emission spectra for FITC. In the example of embedding and electron microscopy (EM) shown in **Figure 3**, spores of the human opportunistic pathogen *Aspergillus fumigatus* were germinated in nutrient broth to allow the development of young hyphae. Following washing to remove excess nutrient solution, the tissue was embedded in LR white, the standard resin for gold labeling of fungi. The gold labeling protocol that follows is best suited for the detection of protein or glycoprotein antigens on the cell surface or in the cell wall, as the fixation procedure described does not adequately preserve the structural integrity of internal endomembrane systems to the standard required to assign subcellular localization of the antigens studied. For preservation of internal endomembranes and associated antigens, preservation procedures based on cryofixation should be used. These include plunge freezing, spray freezing, propane jet freezing, cold metal block freezing and high-pressure freezing¹⁴. Clearly, such techniques are only available in specialized laboratories, and so the inexperienced researcher will require assistance from a skilled electron microscopist who has access to such facilities. A number of facilities do provide cryofixation as a service, such as Brookes Microscopy Consultancy in the BioImaging Unit of the School of Biological and Molecular Sciences at Oxford Brookes University, UK. This facility was recently used by our group

REAGENT SETUP

Blocking buffer for IF Prepare a 0.3% (wt/vol) solution of blocking buffer by dissolving the casein in PBST buffer. Sodium azide can be added as a preservative at 0.05% (wt/vol). ▲ **CRITICAL** It is important to prepare the blocking buffer before the staining procedure as the casein takes a long time to dissolve, typically several hours. Leave the solution overnight for use the following morning.

AP substrate solution for use in IES Dissolve 2.5 g of naphthol-AS-phosphate in 2.5 ml of *N,N*-dimethylformamide. Add this to 500 ml of 0.2 M Tris buffer (pH 9.1) and store in the dark until needed. Just before use, add the Fast Blue BB salt at a concentration of 1 mg ml⁻¹.

IEM fixation buffer Freshly prepare 0.2 M phosphate buffer (pH 7.2) and 10% (wt/vol) paraformaldehyde and 25% (wt/vol) glutaraldehyde solutions. For the paraformaldehyde solution, dissolve 2.0 g of powder in 20 ml dH₂O and heat to 60–65 °C in a fume cupboard. Add a few drops of 1 M NaOH until the solution clears and allow the solution to cool. To prepare the fixation buffer, mix together 5 ml of phosphate buffer, 2 ml of paraformaldehyde solution and 1 ml of glutaraldehyde solution and add 2 ml of dH₂O to bring the volume to 10 ml. The resultant fixative solution contains 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M buffer. Just before use, add 0.05% (vol/vol) Tween 20.

Blocking buffer for gold labeling in IEM Make a 1% (wt/vol) solution of blocking buffer by dissolving the BSA in PBST buffer. Filter the solution through a 0.2-µm sterile filter to remove any insoluble material.

Uranyl acetate and lead citrate solution for section contrasting in IEM

Prepare 2% (wt/vol) solutions of uranyl acetate and lead citrate solutions.

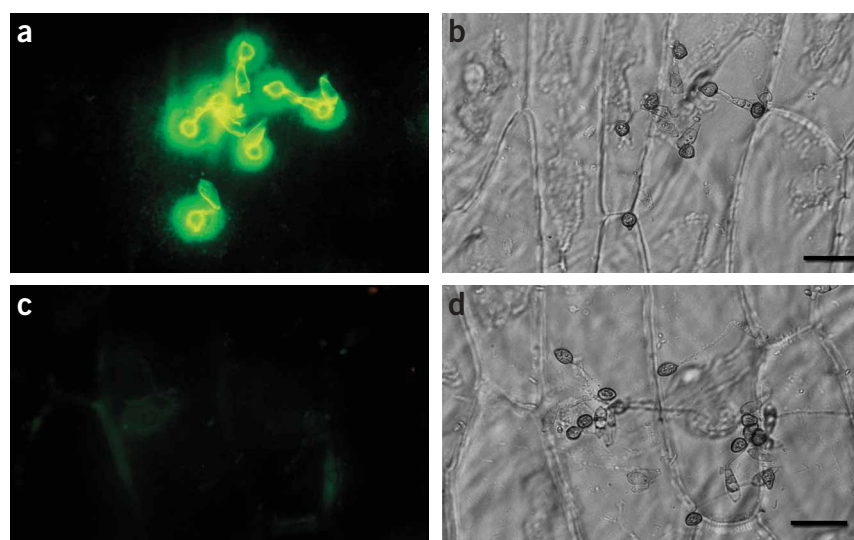


Figure 1 | IF of the rice blast fungus *M. grisea* on the surface of onion epidermis. (a) Sample probed with a mouse mAb specific to spore tip mucilage followed by goat anti-mouse FITC conjugate. (b) Same sample as a, but viewed under bright-field microscopy. (c) Sample probed with TCM control followed by goat anti-mouse FITC conjugate. (d) Same sample as c, but viewed under bright-field microscopy. Scale bars, 20 µm.

PROTOCOL

for the preservation of intracellular membrane structures during gold labeling of an epitope-tagged protein in conidia of the rice blast fungus *M. grisea*¹⁵.

▲ CRITICAL STEP Do not allow the sample to dry out before the addition of the reagents. The sample should be kept in a moist environment at 23 °C throughout the staining procedure. Placing samples in a sealed box with moistened filter paper is sufficient.

! CAUTION Care should be taken throughout the procedure not to damage the sample with forceps or pipette tips.

(A) Immunofluorescence

- (i) Block the sample for 1 h at 23 °C with sufficient blocking buffer to cover the whole sample.
- (ii) Carefully remove the blocking buffer with a pipette and apply sufficient mAb TCS, or TCM control, to cover the whole sample. Incubate in a moist environment for 2 h at 23 °C.
- (iii) Carefully remove the mAb or TCM solutions with a pipette and wash the sample three times (5 min each) with PBST buffer, carefully removing the buffer each time with a pipette.
- (iv) Apply the secondary antibody–FITC conjugate at a 1:40 dilution in blocking buffer and incubate for 1 h at 23 °C, with sufficient volume to cover the whole sample. The choice of secondary fluorochrome conjugate is governed by a number of factors including availability of commercial conjugates. Fluorochromes vary with respect to the signal intensity they provide for each binding site. As a general rule, use the brightest fluorochrome with the most sparsely expressed target antigen. FITC is the most readily available fluorochrome conjugated to antibodies and generates a good signal readily detected by most epifluorescence microscopes and is therefore a good first choice. Other fluorochrome conjugates should be diluted as for FITC.

▲ CRITICAL STEP The secondary antibody–FITC conjugate solution should be made fresh just before use. For *in vivo* studies such as this, it should be diluted in blocking buffer. For studies *in vitro*, where the samples may have been fixed to a glass slide, the fluorochrome conjugate should be diluted in PBS. All incubation steps with FITC conjugates should be performed in the dark by wrapping the box in tin foil.

- (v) Carefully remove the secondary conjugate solution and wash the sample with PBST as described.
- (vi) Apply PBS-glycerol mounting solution to the sample and carefully overlay with a glass coverslip.
■ PAUSE POINT Once the sample is mounted in mounting solution, it can be stored in the dark at 4 °C for several weeks. However, it is advised to examine the sample at the earliest.
- (vii) Examine the sample under UV illumination.

▲ CRITICAL STEP FITC bleaches rapidly under UV exposure. Anti-bleach mounting solutions can be used such as Vectashield. However, experience shows that little is gained by use of such agents. A better approach is to view the sample under low magnification ($\times 10$ objective), under UV, and to rapidly screen for fields of view that exhibit strong fluorescence. The magnification should then be increased ($\times 40$ objective or oil immersion), the image focused under bright-field illumination and the image recorded. The same image can then be taken under UV. Image capture is greatly facilitated if the microscope is fitted with a digital camera. The camera software will calculate appropriate exposure times and publication-grade images captured. For manually operated systems that rely on 35-mm cameras, the most appropriate film for recording images is Kodak Ektachrome 320T (Tungsten) color reversal film. For 35-mm film, exposure times under bright-field optics should be varied. Exposure times under UV will depend on fluorescence intensity, but exposure times of up to 20 s may be required for weaker signals.

(B) IE staining

- (i) Apply sufficient mAb TCS, or TCM control, to cover the whole sample. Incubate in a moist environment for 1 h at 23 °C.

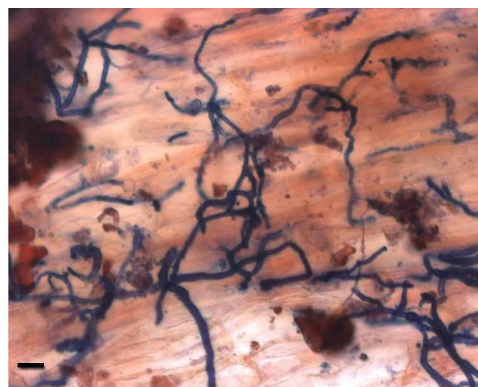


Figure 2 | IES of the soil-borne fungus *T. hamatum* on the surface of a lettuce root. The extracellular antigen was localized with a *Trichoderma*-specific mouse mAb¹. Scale bar, 20 μ m.

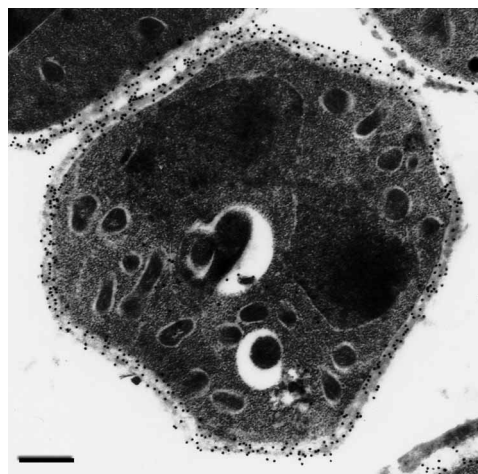


Figure 3 | Gold labeling of an abundant cell wall glycoprotein in the hyphal cell wall of the human opportunistic pathogen *A. fumigatus*. The mAb used to detect the glycoprotein was raised against soluble antigens from hyphae of the fungus. Sections were probed with a mouse mAb followed by a goat anti-mouse 20 nm gold conjugate. Scale bar, 300 nm.

- (ii) Carefully remove the mAb or TCM solutions with a pipette and wash the sample three times (5 min each) with PBST buffer, carefully removing the buffer each time with a pipette.
- (iii) Apply the secondary antibody–AP conjugate at a 1:2,000 dilution in PBST and incubate for 1 h at 23 °C, with sufficient volume to cover the whole sample.

▲ **CRITICAL STEP** The secondary antibody–AP conjugate solution should be made fresh just before use. For plant material, secondary peroxidase conjugates and associated substrates cannot be used because of assay interference from endogenous plant peroxidases.

? **TROUBLESHOOTING**

- (iv) Carefully remove the secondary conjugate solution with a pipette and wash the sample with PBST.
- (v) Apply the substrate solution and incubate in the dark. ▲ **CRITICAL STEP** The length of the substrate step needs to be carefully monitored so as to avoid over-staining. This step should never exceed 30 min.
- (vi) Apply PBS-glycerol mounting solution to the sample and carefully overlay with a glass coverslip.
- (vii) Examine the sample by light microscopy.

■ **PAUSE POINT** If it is not possible to view the samples immediately, they can be stored in the dark at 4 °C in a moist environment for several weeks.

(C) Electron microscopy

- (i) Immerse the tissue in IEM fixative buffer and incubate at 23 °C for 2 h (fungal tissue only) or for 3 h (plant material with fungi).

? **TROUBLESHOOTING**

- (ii) Wash the material two times (10 min each) in 0.1 M phosphate buffer (pH 7.2).
- (iii) Dehydrate the material in a graded water:ethanol series. Immerse the tissue for 5 min in 30% (vol/vol) ethanol, followed by immersion in 50, 75, 95 and 100% ethanol (5 min each).
- (iv) Transfer the tissue to LR White resin and allow infiltration of the resin for 16 h at 23 °C. LR White resin is the standard resin for immunocytochemistry in fungi.
- (v) Transfer the tissue to fresh resin held in gelatine capsules (filled to exclude air) and polymerize the resin by exposing the capsules to UV light for 48 h. Place the light source approximately 20 cm from the capsules.
- (vi) Cut ultra-thin sections of material (70–90 nm thick) using a diamond knife fitted to the ultramicrotome and transfer sections to Piloform-coated nickel grids. This part of the protocol requires collaboration with an electron microscopist trained in the preparation of tissue sections for EM. Other supports can be used such as gold or gold-covered copper grids. However, nickel grids are a cheaper and equally good alternative to these more expensive alternatives.
- (vii) Block the grids by incubating in 50 µl of sterile filtered PBST-BSA blocking buffer for 10 min. During the gold labeling of EM sections, grids should be agitated by vibration from a small air pump. A common item used for this purpose is a fish tank aeration pump on which strips of Parafilm are placed. Because of the small volumes used in the labeling process, gently slot the grids into beads of liquid prepared on strips of Parafilm.

! **CAUTION** Nickel grids are electrostatic and extremely fragile. Great care should be taken when handling the grids. This can only be achieved by using a dedicated set of very sharp anti-magnetic tweezers.

- (viii) Wash the grids three times (3 min each) in PBST washing buffer that has been filtered through a 0.2 µm filter to remove particulate matter.
- (ix) Incubate the grids in mAb TCS or TCM control for 1 h at 23 °C. Because of variability in affinities and specificities of antibodies, it is advised to test a range of antibody concentrations to maximize the signal and minimize background labeling. For mAbs, 1:100 dilution of TCS in TCM should be tried. For polyclonal antisera, 1:10 to 1:10,000 dilutions of sera in PBST blocking buffer should be used.
- (x) Wash the grids four times (3 min each) in PBST washing buffer.
- (xi) Label the sections for 1 h at 23 °C, by incubating the grids in 50 µl of secondary antibody-colloidal gold conjugate diluted 1:20 with PBST blocking buffer. As with primary antibodies, the concentration of the secondary gold conjugate may need to be adjusted in the event that nonspecific binding of the gold conjugate is apparent. However, we have found that a 1:20 dilution of the conjugate gives maximum signal with minimal nonspecific background labeling.

? **TROUBLESHOOTING**

- (xii) Wash the grids four times (3 min each) in PBST washing buffer and place the grids on Whatman filter paper to dry at 23 °C.

■ **PAUSE POINT** At this point, the grids can be stored in a desiccator at 23 °C before section contrasting.

(D) Section contrasting with uranyl acetate and lead citrate

- (i) Incubate the grids for 20 min in uranyl acetate followed by lead citrate for 4 min. Return the dried grids to a dessicator before observation using a transmission electron microscope at 80 or 100 kV.

● TIMING

Step 1A, IF, 5 h

Step 1B, IES, 4 h

Step 1C(i–vi), tissue embedding, 4 days

Step 1C(vii–xiii), gold labeling and section contrasting, 4 h

? TROUBLESHOOTING

Timing is critical in immunoassays. Once an immunoassay is started, the entire procedure must be continued to completion. Blocking buffers vary in the type of blocking agents used and the concentrations employed. Other blocking agents typically used in immunoassays are BSA and gelatin. These can be used in place of casein should the signal strength be weaker than anticipated. However, in the case of plant material, casein is the most appropriate blocker to start with. The concentration of the blocker used in the immunoassay will need to be determined empirically.

Preparation of AP secondary conjugates

Unlike IF, where the secondary FITC conjugate should always be used at a 1:40 dilution, the concentration of AP conjugates will need to be tested empirically for each antibody–antigen interaction, but a good starting concentration is 1:1,000 or 1:2,000.

Preparation of IEM fixative

The IEM fixative uses phosphate buffer and glutaraldehyde supplemented with formaldehyde. Phosphate buffers are more physiological than any other buffer, because they are found in living systems in the form of inorganic phosphates and phosphate esters. Phosphate buffers stabilize the pH of the fixative more efficiently than any other buffer. The pH of the buffers changes little at different temperatures and they can be stored for several weeks under cold conditions. Despite these attributes, phosphate buffers are not always desirable and alternatives such as HEPES^{16,17} and PIPES^{16,17} should be used, although these buffers may also not be suitable for every type of study. For studies with fungi, we have found that phosphate buffers are the most suitable buffers for the preparation of IEM fixative and subsequent gold labeling studies and should therefore be used in the first instance. The antigenicity of proteins is usually inversely proportional to the level of aldehyde crosslinking; so low concentrations of glutaraldehyde, supplemented with 1–4% formaldehyde, are usually recommended for immunocytochemistry. Nevertheless, crosslinking must be sufficient to avoid diffusion or extraction of antigens. We have found that the concentrations of glutaraldehyde and formaldehyde used in this protocol satisfy both requirements, but different concentrations of glutaraldehyde and formaldehyde can be used¹⁴. As with all antibody–antigen interactions, optimum conditions for antigenicity and preservation of cell structure are best established empirically.

Choice of colloidal gold particle size for EM labeling studies

For gold labeling of sections for EM, different-sized gold particles can be used. If no labeling is found with a 20 nm gold conjugate, 10 or 1 nm gold conjugates can be used. If no labeling is found with either of these, it should be assumed that the primary antibody (monoclonal) is unable to bind to its epitope. Detection of an antigen at the IF and IES level does not automatically assume suitability of a mAb for EM gold labeling studies.

ANTICIPATED RESULTS

Localization of antigens using FITC-labeled antibodies is one of the most sensitive immunoassay systems, as it relies on the detection of photons. This results in cells with bright green fluorescence such as that illustrated in **Figure 1a**. Clearly, the intensity of fluorescence will vary according to the affinity (strength of binding) of the antibody for its target epitope, the avidity (the intrinsic affinity of the antibody for the epitope, the valency of the antibody and antigen, and the geometric arrangement of the interacting components) of the antibody–antigen interaction and the degree of expression of the antigen. In the absence of antibody binding, for example in negative control samples, no fluorescence should be observed (**Fig. 1c**). In cases where autofluorescence interferes with FITC-labeled detection systems (root tissue and uncleared leaf material are typical examples), FITC can be replaced with an alternative fluorochrome such as Texas Red or Alexa Blue. However, Alexa Blue emits a dim signal and therefore is suitable only with the most densely expressed antigens. Texas Red is particularly useful where dual labeling is required. For example, Gilbert *et al.*¹⁵ used a dual GFP and Texas Red anti-V5 epitope tag procedure to colocalize a P-type ATPase with Golgi protein in conidia of the rice blast fungus *M. grisea*. In situations where IF localization of antigens is not possible, bright-field microscopy can be employed using IES procedures (**Fig. 2**). We have found that it can be an extremely sensitive alternative to IF where autofluorescence is an issue. Unlike IF and IES, antibody labeling of material in electron microscope sections permits the visualization of intracellular, extracellular and cell wall-bound antigens. Intense gold labeling is often observed (**Fig. 3**) but, as with IF, the intensity of staining depends on affinity, avidity and the degree of antigen expression. In control samples, no gold labeling should be observed. A caveat to all of these assays is the suitability of any given antibody to a chosen immunoassay format. It is often the case that an antibody performs well in one assay but fails in

another. However, a general rule to follow is that if an antibody performs well in IF and IES, it should also perform well in EM gold labeling studies. However, as with all biological systems, there are exceptions to every rule.

COMPETING INTERESTS STATEMENT The authors declare that they have no competing financial interests.

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