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A PREPARATION PROTOCOL FOR POSTEMBEDDING IMMUNOELECTRON MICROSCOPY OF *Dictyostelium discoideum* CELLS WITH MONOCLONAL ANTIBODIES

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Abstract

On-section-labelling of *Dictyostelium discoideum* cells poses severe problems in retaining adequate morphology and antigenicity. Monoclonal antibodies are an essential tool in biochemistry and molecular biology because of their specificity and low background staining. Unfortunately their advantage, the recognition of only one distinct epitope, is often a handicap for immunoelectron microscopy. Resin embedding and steric hindrance of the gold-tagged secondary antibodies further reduce the efficiency in detecting antigens making the localization of less abundant antigens difficult if not impossible. A successful preparation protocol should retain morphology and antigenicity, allow the antibodies easy access to the antigen and use a detection system which visualizes as many of the primary antibodies as possible. Fixation of *Dictyostelium* cells with buffered formaldehyde and picric acid, cryosectioning and the use of ultra-small gold conjugates enabled us to label most of the antigens under investigation.

Key Words: Cryoultramicrotomy, on-section-labelling, monoclonal antibodies, ultra-small gold conjugates, silver enhancement, autometallography, *Dictyostelium discoideum*.

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Introduction

Immunogold labelling at the electron microscopical level has become an indispensable tool in cell biology. Nevertheless, in the wide field of *Dictyostelium discoideum* research only a few authors have demonstrated on-section-labelling: spore coat proteins (Devine et al., 1983); discoidin I (Cooper et al., 1986); secretory glycoproteins (West & Erdos, 1988); stalk-specific proteins (McRobbie et al., 1988); and two components of a protein crystal (Bomblies et al., 1990). *Dictyostelium discoideum* has proven to be a difficult organism for electron microscopy. Retaining ultrastructural details at an electron microscopical level poses severe problems. The addition of 1% glutaraldehyde to *Dictyostelium* cells immediately stops pseudopode formation and the membrane becomes rigid. However, vesicles and organelles still continue to move around and they only slowly come to a rest (unpublished observation): the fixation process is too slow. The penetrating aldehyde changes the intracellular osmotic conditions which leads to rupturing of vesicles and releasing of proteases. The cytoplasm will be partially digested before the fixative arrests the process. In the electron microscope the cells have lost most of their ultrastructural details. Only the introduction of a fixation protocol using osmium tetroxide and glutaraldehyde simultaneously (Schwarz, 1973) leads to excellent ultrastructural appearance, i.e. without signs of autolysis, of *Dictyostelium discoideum* cells after Epon embedding. Unfortunately strong fixation, which includes osmium tetroxide, is unfavourable for antigenicity (for an exception see Cooper et al., 1986). Therefore, it is necessary to find fixatives that penetrate rapidly into *Dictyostelium* cells but do not destroy antibody recognition sites.

Epon is not very favourable for immunolabelling because of its high reactivity with cellular macromolecules (Causton, 1986). Therefore, the samples should

preferentially be prepared for cryosectioning according to Tokuyasu (1973) or by progressive lowering of temperature (PLT) according to Carlemalm et al. (1982).

Using monoclonal antibodies, the number of epitopes is limited which results in reduced labelling. An additional loss of signal is due to steric hindrance of the gold-tagged secondary antibodies. As a result, rare antigens are difficult to visualize. The introduction of the ultra-small gold conjugates (Janssen Life Science) combined with silver enhancement (Danscher, 1981; Stierhof et al., 1991) remarkably improved the sensitivity.

We have studied the localization of the following proteins in *Dictyostelium discoideum*. The crystal protein (Humbel et al., 1989; Bomblies et al., 1990) is the major component of a protein crystal enclosed in rough endoplasmic reticulum of *Dictyostelium discoideum* cells. It is developmentally regulated and has sequence similarities with esterases. We chose the crystal protein as a model system because the target of the antibodies, the crystals, are easily seen and the antigen is concentrated in a restricted area. After the preparation and labelling conditions had been established, we checked them on two proteins which are less concentrated and more difficult to label: the contact site A protein and hisactophilin. The contact site A protein is a cell surface glycoprotein which mediates cell-cell contact of individual *Dictyostelium* cells during the aggregation phase (Müller & Gerisch, 1978). We used an antibody which recognizes the protein moiety of the contact side A protein to avoid cross-reactivity with other carbohydrates (Bertholdt et al., 1985). Hisactophilin is a 17 kD F-actin-binding protein. It has been shown by immunofluorescence that hisactophilin is concentrated at the plasma membrane (Scheel et al., 1989).

In this paper, we describe a preparation protocol which allows protein localization with monoclonal antibodies on ultrathin sections of *Dictyostelium discoideum* cells.

Materials and Methods

Cultivation of *Dictyostelium discoideum*

Cells of *Dictyostelium discoideum* strain AX2-214 were cultivated in liquid nutrient medium and harvested at a density of not more than 5 x 10^6 cells per ml (Malchow et al., 1972). Washed cells were examined immediately (vegetative cells), or starved for 6 h in 17 mM Sϕrensen buffer, pH 6.0, at a density of 1 x 10^7 cells per ml (aggregation competent cells).

Fixation

Vegetative cells were fixed in suspension, whereas starved cells were seeded on a petri dish to allow aggregation before fixation. The aggregates are easily harvested from the petri dish as their contact with the substrate is very weak. The following fixation protocols were used:

a) 0.5%, 1%, 2%, 4% formaldehyde in Sϕrensen buffer pH 6.0 for 15 min each step, followed by 8% formaldehyde for 1 h.

b) 1% formaldehyde with a low concentration (0.02%) of glutaraldehyde in Sϕrensen buffer pH 6.0, for 1 h.

c) 15% of a saturated aqueous solution of picric acid and 2% formaldehyde (Stefanini et al., 1967) in 10 mM PIPES, pH 6.0, for 1 h.

Formaldehyde was always freshly prepared by depolymerizing paraformaldehyde and the mixtures of the fixatives were made shortly before use. Fixation was done at room temperature.

Further processing

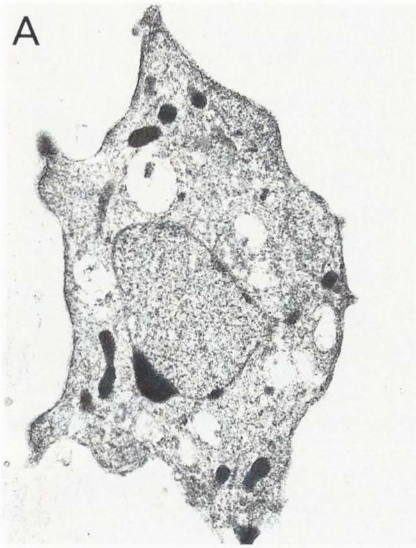
The fixed cells were embedded in 10% gelatin for better handling and cubes of about 1 mm^3 were cut. One portion of the cubes were infiltrated with 2.3 M sucrose for cryosectioning according to Tokuyasu (1973). Usually infiltration took place in tubes for 4 h. The criteria for sufficient infiltration was sedimentation of the cubes to the bottom of the tube. The sucrose-infiltrated cubes were mounted on a holder and frozen in liquid nitrogen, in which the samples were stored until use. Sections were cut with the cryoultramicrotome (Ultracut/FC4, Reichert-Jung, Vienna) at about -120°C.

The other portion of the cubes was dehydrated in a graded series of ethanol while lowering the temperature to -30°C. Then they were embedded at the same temperature into either Lowicryls K4M, K11M, HM20, or HM23 (Carlemalm et al., 1982; Acetarin et al., 1986). The cubes were impregnated sequentially with a mixture of ethanol and resin (1 + 1, 1 + 2) and 100% resin for 1 h at each step, 100% resin overnight and an additional change with 100% resin for 1 h. The samples were polymerized under UV irradiation at -30°C as described by Humbel & Müller (1986).

Immunolabelling

Monoclonal antibodies against the protein portion of the contact site A, hisactophilin and the crystal protein were produced and characterized as described previously (Bozzaro, 1985; Bertholdt et al., 1985; Scheel et al., 1989; Humbel et al., 1989; Bomblies et al., 1990). All

Figure 1. Cryosections of *Dictyostelium discoideum* cells after different fixation protocols: **A, B** formaldehyde, **C, D** formaldehyde/glutaraldehyde, and **E, F** formaldehyde/picric acid. Independent of the fixation protocol used, the cells are well preserved and not degraded by autolysis. Bar represents 2 µm (A, C, E) and 500 nm (B, D, F).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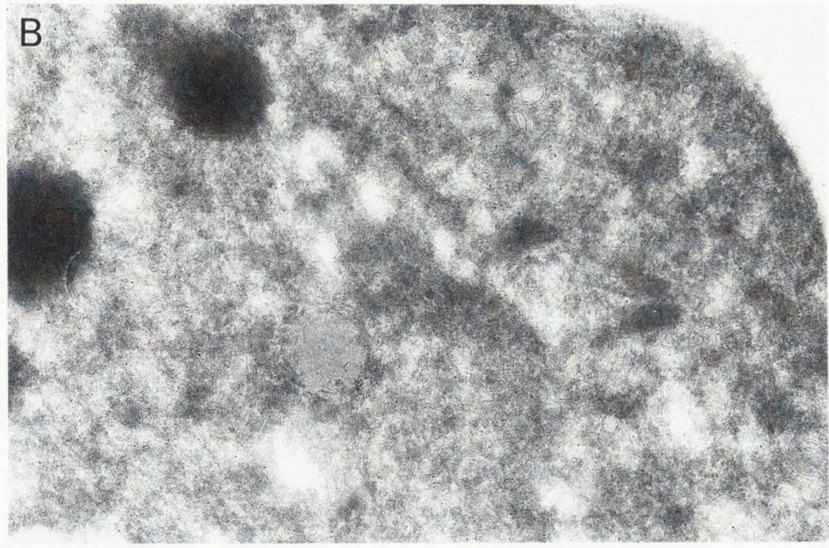
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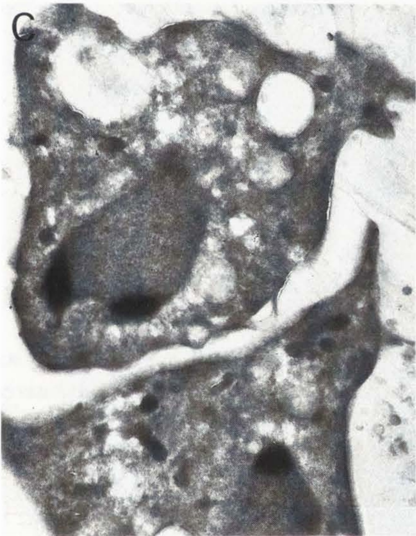
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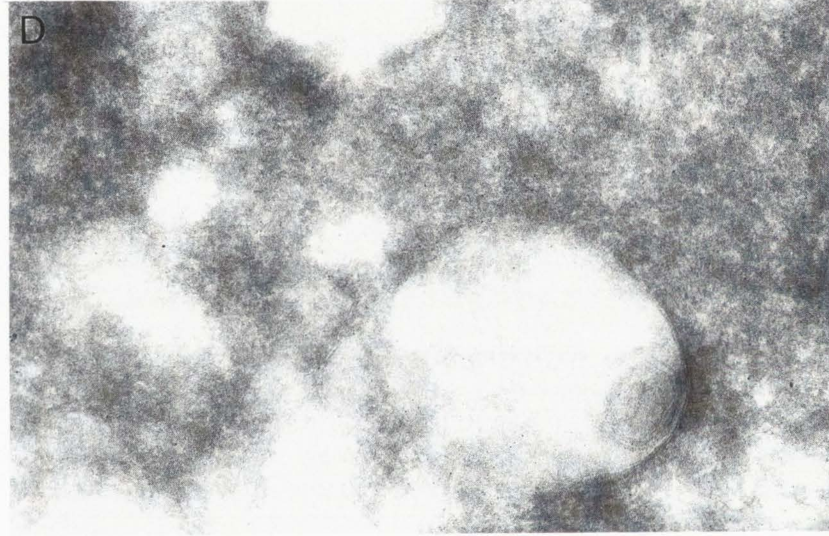
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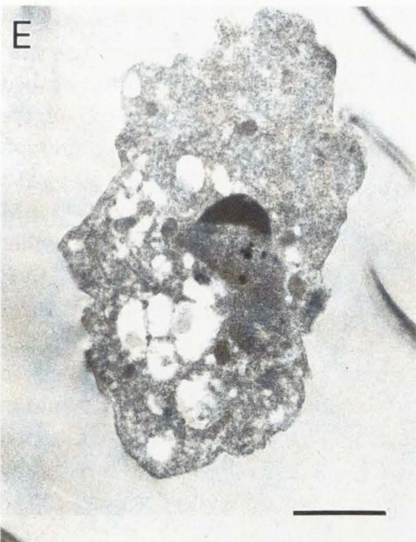
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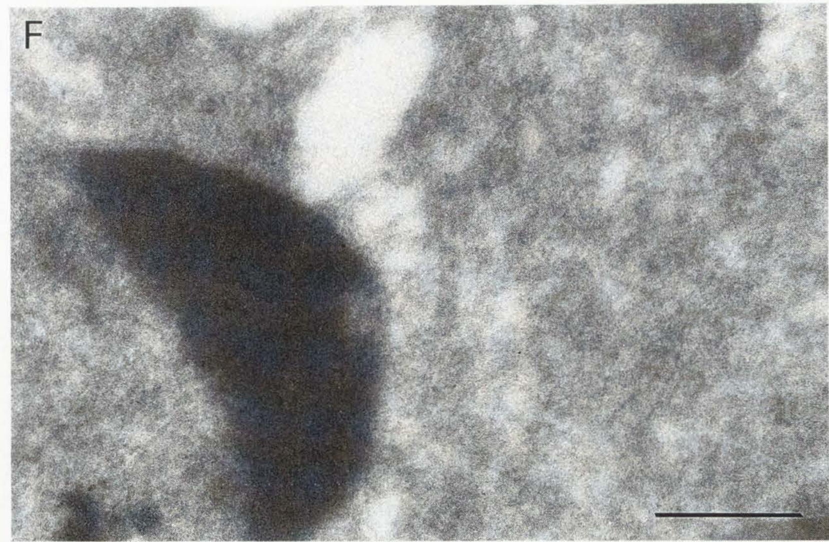
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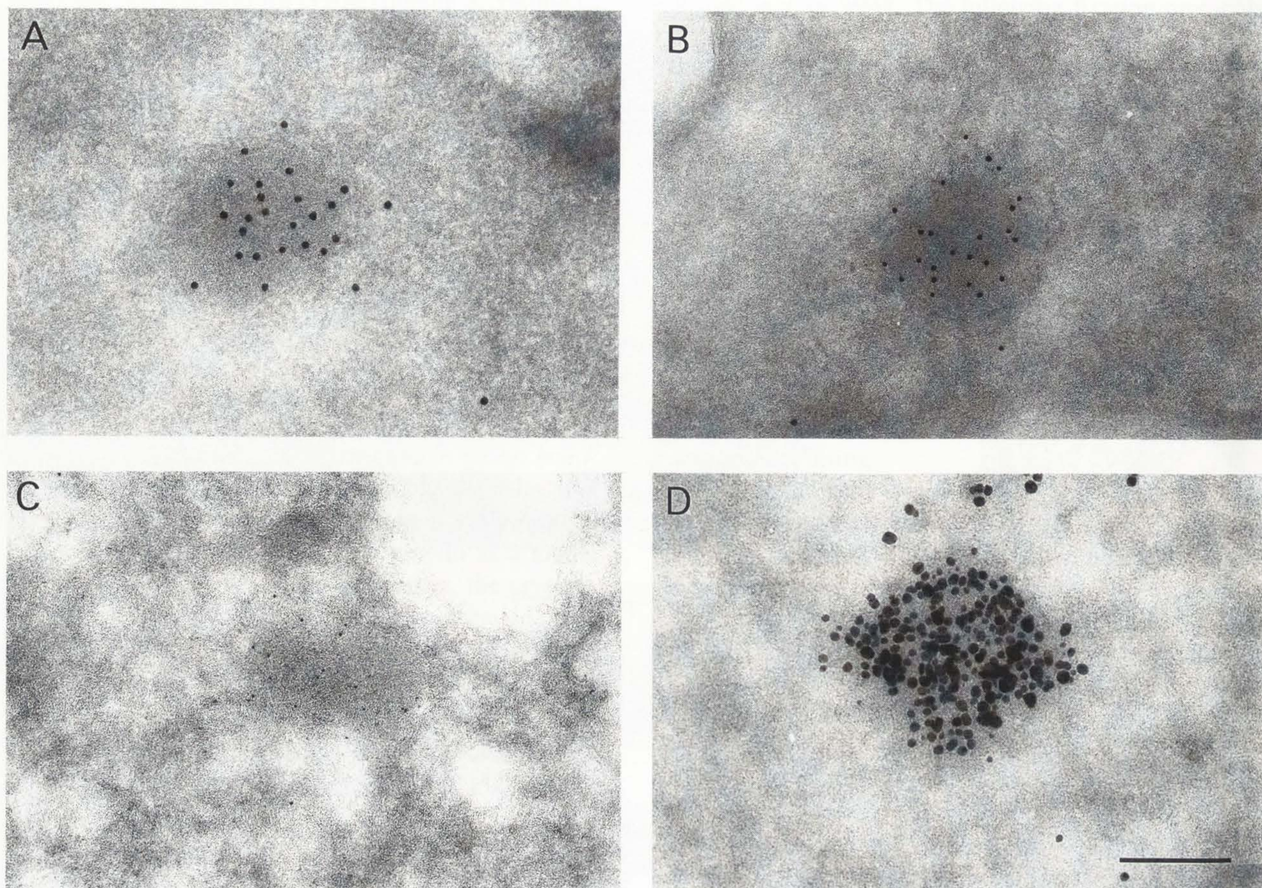

Figure 2. Cryosections of formaldehyde/picric acid fixed cells of *Dictyostelium discoideum* labelled against the crystal protein. The primary antibodies were visualized with gold-tagged secondary antibodies with different particles sizes. **A**: 15 nm, **B**: 10 nm, **C**: 5 nm and **D**: silver-enhanced ultra-small gold particles. There is no obvious difference in labelling efficiency if gold particles of 5 nm and larger were used (A-C). Labelling with ultra-small gold particles, however, resulted in a prominent labelling (D). Bar represents 200 nm.

antibodies were tested on semithin cryosections (0.2 µm to 0.5 µm) and examined by immunofluorescence microscopy before they were used in electron microscopical investigations. The cryo- or Lowicryl sections were collected on Pioloform and carbon coated gold grids. They were incubated for 5-10 min on PBS containing 100 mM glycine to inactivate free aldehyde groups, then on PBS containing 0.05% fish gelatin (Birrell et al., 1987) and 0.5% bovine serum albumin to mask unspecific protein binding sites (Van Bergen en Henegouwen & Leunissen, 1986). The primary antibodies were diluted to a concentration of 1 µg/ml to 5 µg/ml in PBS containing the blocking proteins (Schwarz & Humbel, 1989). They were detected by secondary goat anti-mouse antibodies conjugated to colloidal gold of different sizes (BioCell, Cardiff, United Kingdom; Janssen Life Sciences, Beerse, Belgium). In order to visualize the ultra-small gold particles they were enlarged by silver deposition as described by Stierhof et al. (1991). The sections were postfixed for 10 min with 1% glutaraldehyde because the silver enhancer has a pH of 3.5 which favours dissociation of the antibodies. The sections are carefully washed with bidistilled water to avoid autoprecipitation of the silver ions. Immediately before use 600 µl of a solution of 25% gum arabic, 100 µl 2 M citrate buffer, 150 µl 515 mM hydrochinone in bidistilled water, and 150 µl 37 mM silver lactate in bidistilled water are mixed. For further details confer Danscher (1981) and Stierhof et al. (1991, 1992). The grids are incubated on drops of the enhancer at 20°C for 20 min.

After enhancement the sections are washed in bidistilled water. Cryosections were stained for 10 min in 2% neutral uranyl acetate, 1 min in 2% aqueous uranyl actetate and embedded in methylcellulose as described by Tokuyasu (1978). Lowicryl sections were stained for 10 min in 2% aqueous uranyl acetate.

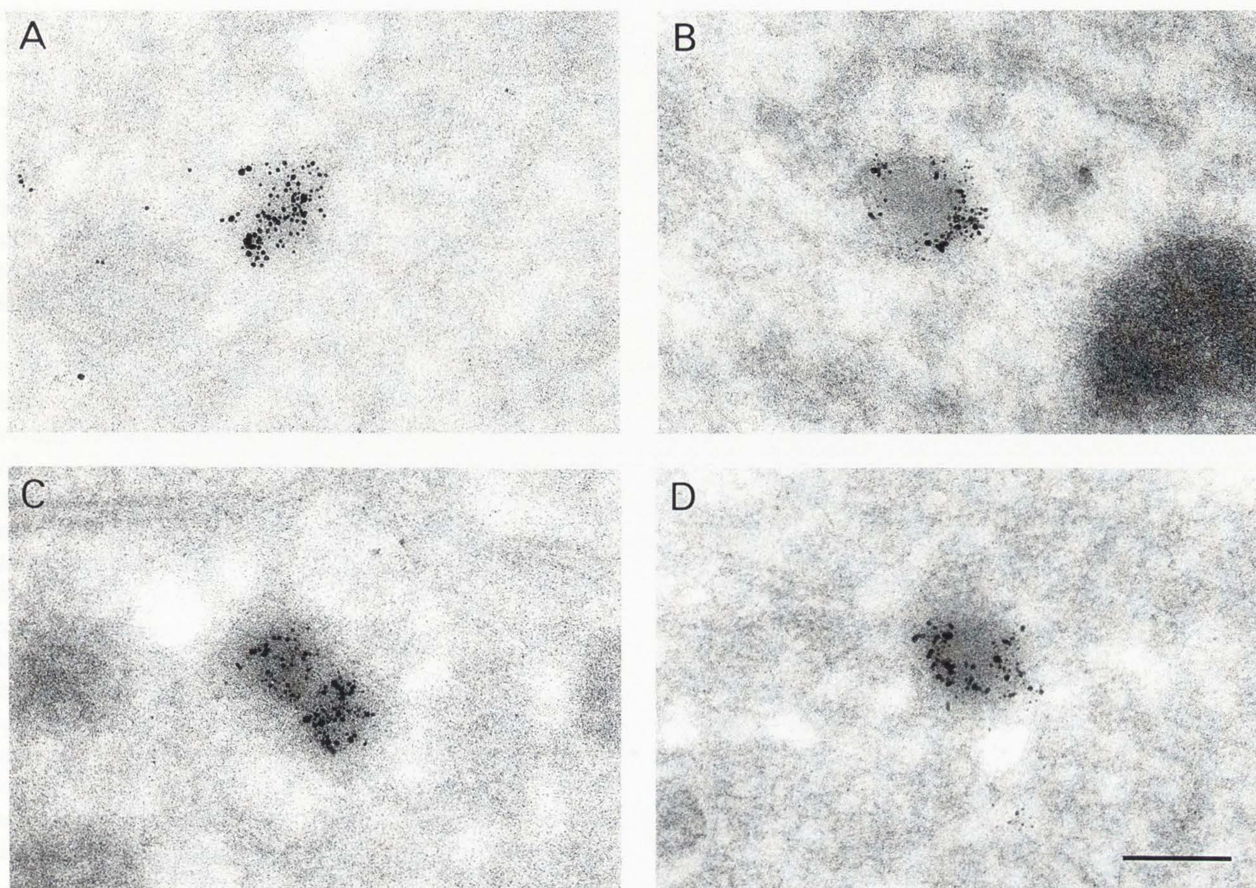

Figure 3. Sections of *Dictyostelium discoideum* cells embedded in different Lowicryl resins labelled for the crystal protein and visualized with silver enhanced ultra-small gold-tagged secondary antibodies. **A**: K4M, **B**: HM20, **C**: K11M, **D**: HM23. Irrespective of the resin used, the labelling efficiency is reduced compared to that on cryosections. Bar represents 200 nm.

Results

Morphology

The ultrastructure of cryosectioned *Dictyostelium discoideum* cells is well preserved irrespective of the fixatives used (Fig. 1). Our main criterion was a dense cytoplasm indicating that no autolysis had taken place. Nevertheless, there are some important differences between the fixatives which should be mentioned. The formaldehyde fixation leads to a granular appearance of the cytoplasm and to a considerable shrinkage of the cells (Fig. 1A, B). A gradual increase in the formaldehyde concentration was important to prevent a total collapse of the cells. Mixtures of formaldehyde and glutaraldehyde or formaldehyde and picric acid gave superior results. Overall shrinkage is reduced, the cytoplasm is more homogeneous and membranes are clearly visible (Fig. 1C-F). The formaldehyde/picric acid fixation is preferable because of better reproducibility. The different fixation protocols did not influence the labelling efficiency of the antibodies tested (data not shown).

The same results were obtained with Lowicryl embedded cells (Fig. 3).

Influence of the size of the gold colloids

Using different sizes of gold particles, ranging from 5 nm to 15 nm, did not always give clear-cut results. Especially in cases of an unknown distribution of an antigen, two to three gold particles at one site are not sufficient convincing evidence for a specific location of the protein in question. Monoclonal antibodies specifically recognize one particular epitope and often only a few epitopes are accessible to the antibodies. Therefore, we need a detection system which visualizes as many of the primary antibodies as possible. To monitor the difference in recognition of primary antibodies by secondary gold-tagged antibodies with different particle sizes, we labelled ultrathin cryosections for the crystal protein (Bomblies et al., 1990). Individual crystals are easily detected, they represent a sufficient amount of antigen, i.e. epitopes, and

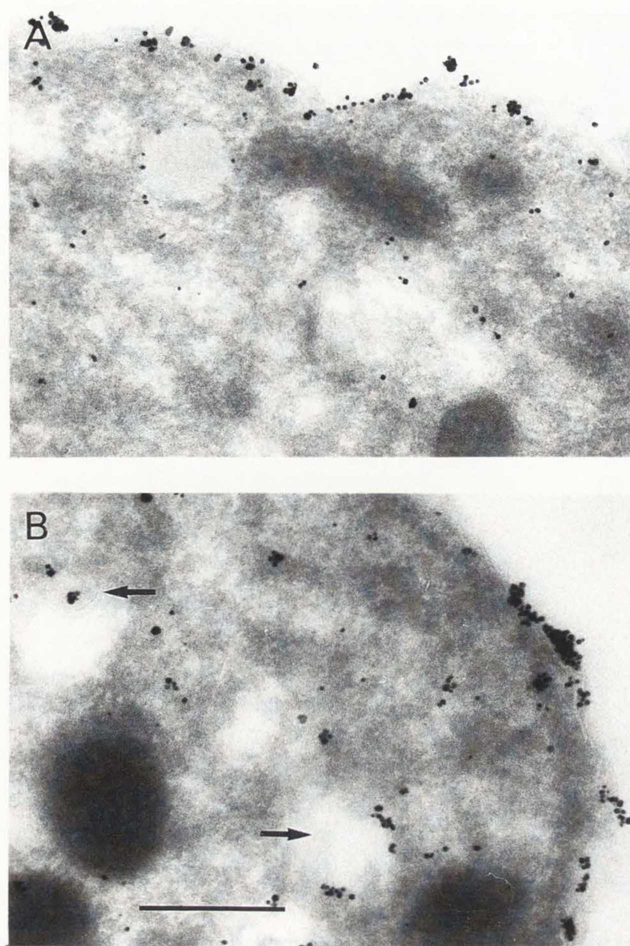

Figure 4. Cryosections of *Dictyostelium discoideum* cells labelled for hisactophilin (**A**) and contact site A protein (**B**), visualized with silver enhanced ultra-small gold tagged secondary antibodies. **A**: Hisactophilin, a F-actin binding protein, is predominantly localized at the plasma membrane. **B**: The contact site A, a membrane protein, was not only found in the plasma membrane but also in transport vesicles (→). Bar represents 500 nm.

there is an excellent monoclonal antibody available. The labelling procedures used for all sections were identical (Fig. 2). There was no large difference in labelling efficiency if gold particles of 15 nm to 5 nm were used. Using silver enhanced ultra-small gold particles, however, clearly gave a more intense labelling (Fig. 2D). The more efficient detection of primary antibodies by the ultra-small gold probe opens the possibility to get clearer labelling results for less abundant antigens as, for example, hisactophilin (Fig. 4A).

Lowicryl embedding

Resin embedded samples have some advantages compared to preparations for cryosectioning: 1) the specimens are easily stored to establish a library of cells grown under different culture conditions or of different mutants; 2) sectioning is performed at ambient temperature without special equipment; 3) it is possible to make large sections or serial sections for 3D analysis. Therefore, embedding following the lowering of temperature method according to Carlemalm et al. (1982) into the four different Lowicryl resins was tested. Labelling with our test antibody for the crystal protein gave good results (Fig. 3). Embedding in Lowicryl, however, reduced the labelling efficiency for the monoclonal antibodies tested.

Comparison of cryo- and resin sections

We were able to convincingly demonstrate the distribution of hisactophilin (Fig. 4A), contact site A (Fig. 4B) and our test protein, the crystal protein (Fig. 2) on cryosections, using the ultra-small gold probes. However, only the antibodies against the crystal protein gave good results (Fig. 3) on Lowicryl sections. A slight reduction in efficiency was observed as compared to cryosections. The antibodies against hisactophilin only recognized the antigen at sites where the Lowicryl had come away from the cell membrane. Well embedded parts of the cell membrane were not labelled. The antibodies against the contact site A protein did not react at all on Lowicryl sections (data not shown).

Neither the different Lowicryl resins (Fig. 3) nor the cryosections (Fig. 2D) influenced the quality of silver enhancement.

Discussion

The delicate ultrastructure of *Dictyostelium discoideum* cells is best preserved for postembedding immunolabelling using formaldehyde/picric acid fixation as described by Zamboni & De Martino (1967, Stefanini et al., 1967). The fixatives used are small molecules and penetrate the cells more easily and faster than e.g. glutaraldehyde (Stefanini et al., 1967). The speed of fixation competes successfully with the speed of protein degradation resulting in good morphology.

Our results confirm the observations of Accinni et al. (1974) that fixation with formaldehyde/picric acid has only limited influence on the antigenicity and that immunolabelling is still possible.

A large number of monoclonal antibodies could be tested for their labelling using immunofluorescence microscopy. We used ultrathin or semithin sections (up to 0.5 µm) of samples prepared for electron microscopy e.g. cryosectioning according to Tokuyasu (1973). These samples best reflect the labelling observed at electron microscopical level. The use of a fluorescence probe instead of the gold probe is the only difference between the two techniques. Only those monoclonal antibodies

which gave excellent results in immunofluorescence were used for immunogold labelling. At the electron microscopical level, however, only the monoclonal antibodies recognizing the two components of the protein crystal (Bomblies et al., 1990) gave satisfactory labelling with the commonly used gold-tagged secondary antibodies of a gold size from 5 nm to 15 nm. The discrepancy between fluorescence labelling and gold labelling could largely be overcome using the recently introduced ultra-small colloidal gold probes (Jan Leunissen, Aurion) followed by silver enhancement. The advantage of monoclonal antibodies, their specificity for one particular epitope, often presents a disadvantage for immunolabelling studies. Most likely, only a small number of these epitopes are accessible on sections. Thus a satisfying labelling result can only be obtained if every primary antibody is detected and visualized by the secondary antibody. The loss of sensitivity from the antigen to the final gold particle is about 85% (Howell et al., 1987) for 5 nm protein A-gold. There are two possible explanations for this low detection of antigens: 1) steric hindrance (Howell et al., 1987; Voorhout et al., 1986), a second antibody with a large particle may only attach to a protruding primary antibody or 2) the gold particles repulse each other due to their net negative surface charge. Howell et al. (1987) found that the labelling efficiency decreases significantly for antigens at higher concentrations.

Two to three of the small sized ultra-small gold particles bind to one antibody (Jan Leunissen, personal communication), whereas many antibodies bind to one gold particle of the larger size, resulting in a bulky probe. Therefore, the ultra-small gold probe may penetrate better into the cavities of the surface relief of cryo- and plastic sections (Kellenberger et al., 1987) and find easier access to the antigen. De Graaf et al. (1991) showed in pre-embedding labelling experiments that ultra-small gold particles easily penetrate into the dense meshwork of the nucleus of permeabilized HeLa cells. The net electrical charge on the surface of the gold particles also decreases with decreasing volume and thereby reduces repulsion effects.

To visualize the ultra-small gold particles, however, an efficient silver enhancement step is required. It could be demonstrated, that the method according to Danscher (1981) is best suited to enhance most of the ultra-small particles (Stierhof et al., 1991; 1992).

The limited success of using Lowicryl sections in combination with monoclonal antibodies for immunolabelling is most likely due to a further decrease of exposed epitopes (Kellenberger et al., 1987). Although penetration of ultra-small gold tagged antibodies into well fixed cryosections is limited (Stierhof et al., 1986; Stierhof & Schwarz, 1989), our results strongly support the idea, that epitopes are more accessible to primary antibodies in cryosections than in Lowicryl sections.

The combination of a fast chemical fixation method, which does not seriously interfere with antigen-antibody recognition with cryosectioning and with the use of ultra-small gold probes provides an excellent tool to study the distribution of antigens in *Dictyostelium discoideum* cells with monoclonal antibodies.

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We thank Dr. G. Gerisch for continuous support, Dr. J. L. M. Leunissen (Aurion, Wageningen) for providing ultra-small conjugated anti-mouse antibodies prior to commercial release, Drs. M. Brink, H. Schwarz, Y. D. Stierhof, P. Thomas and A. J. Verkleij for valuable discussions and their help in preparing this article.

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Discussion with Reviewers

P. Walther: The authors mention the slow fixation process using glutaraldehyde. Fastest immobilization, however, is achieved by rapid freezing. Did you try it?

J. A. Hobot: If fixation is not an ideal situation for *D. discoideum*, why was a technique where fixation is not required, like cryosubstitution, not tried? Would the resin

embedding, performed at low temperature, still not allow immunolabelling by the monoclonals? If not, then the possibility arises that these antigenic sites in the tissue are sensitive to the organic solvent and/or the organic resin. Then, in this case, cryoultramicrotomy is definitely needed. Have the authors any data on this point?

Authors: We completely agree that cryofixation followed by low-temperature embedding (Humbel et al., 1983) is the best method of choice and we did try it. With our equipment, i.e. plunge-freezing and freezing with a propane-jet (Müller et al., 1980) it was, however, not possible to cryofix *Dictyostelium discoideum* adequately, i.e. without visible ice crystal formation. *Dictyostelium* probably needs more elaborate techniques for cryofixation. Maybe freezing under high pressure (Müller & Moor, 1984) would be sufficient to vitrify cells of *Dictyostelium*.

In general we prefer cryosections for immunolabelling with monoclonal antibodies. With all monoclonal antibodies, not directed against a carbohydrate epitope, we got more intense labelling on cryosections than on Lowicryl sections which would support the idea that the protein sites are indeed sensitive to the organic solvent and/or the organic resin.

J. A. Hobot: The manuscript draws attention to the possibility of steric hindrance during immunolabelling by colloidal gold tagged to the secondary antibody if its size is larger than 1 nm. Naturally a similar phenomenon would occur if protein-A colloidal gold was used. But what would be the results in terms of labelling sensitivity if small protein-A colloidal gold (3-5 nm) was used? Do the authors have any data on this?

Authors: We only exceptionally used protein-A colloidal gold to detect monoclonal antibodies. With gold particles of 4 nm and larger bound to protein-A we got the same intensity of labelling as with gold-tagged secondary antibodies of gold particles larger than the ultra-small probe. We did, however, never use protein-A colloidal gold smaller than 4 nm in our studies.

G. B. Birrell and O. H. Griffith: Although the magnifications in Figs. 2 and 3 are the same, the labeled regions appear much smaller in Fig. 3. What is the cause of this? Also, the silver-enhanced 1 nm gold particles in Fig. 2D appear to be significantly larger than those in Fig. 3. Were these samples silver-enhanced for the same lengths of time?

Authors: Yes both samples were enhanced for 20 min. The difference in size of the silver-enhanced ultra-small gold particles in Fig. 2D and Fig. 3 is most likely due to a difference in temperature at which the enhancement process took place. The samples in Fig. 3 were processed in a thermo-stated room at 18°C, whereas the samples in Fig. 2 were processed at room temperature, i.e. at around 22°C.

The labelled area does not completely reflect the size of the crystals and as shown in the paper the labelling efficiency of resin embedded material is reduced to cryosectioned material. The length of side of the crystals varies between 100 and 500 nm (Bomblies et al., 1990) with a highest population between 200 and 300 nm. We analyzed a few crystals on cryo- or Lowicryl sections and got the following results: the mean length of side was 235.3 nm ± 83.4 nm (n = 9) on cryosections; 189.5 nm ± 65.1 nm (n = 14) on sections of the polar Lowicryl resins K4M and K11M; and 146 nm ± 31.9 nm (n = 10) on sections of the apolar Lowicryl resins HM20 and HM23. The size of crystals in sections of the apolar resins compared to those in cryosections indeed seem to be smaller by about 30%. It could be possible that the crystals shrink on removing the crystal water in analogy to collapsing of macromolecules during dehydration as described by MacKenzie (1972). Further it could be shown that Lowicryl resins exert large and variable influences on the size of the embedded biological material (Schwarz and Humbel, 1989), e.g. sections of the polar Lowicryl K4M expand during sectioning on the water surface.

Further studies using cryofixation followed by cryosectioning (Dubochet et al., 1987) and/or freeze-substitution in combination with electron diffraction studies are needed to answer this question completely.

Additional References

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