Immuno-Electron Microscopy Techniques: I. Pre-embedding Immunolabelling of Intracellular Antigens Using 1 nm Colloidal Gold Probes and Silver Enhancement

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ABSTRACT

As an alternative procedure to post-embedding immunolabelling in resin-embedded samples, pre-embedding immunolabelling increases the likelihood of antigen recognition by the antibody during immunocytochemical localization. The development of one nanometer colloidal gold conjugated antibodies offers new probes for intracellular pre-embedding immunolabelling. In this report, the use of one nanometer gold probes coupled with silver enhancement was examined in three commonly used sample preparations: extracted cell suspensions, detergent permeabilized cell cultures, and vibratome tissue sections. One nanometer gold probes have greater sensitivity over larger gold probes as demonstrated in the extracted cell preparations. However, intact cells need to be rigorously permeabilized to allow the one nanometer gold probes to reach antigen sites. One nanometer gold probes can penetrate 4-5 μm into vibratome sections treated with detergent permeabilization.

KEY WORDS

Immunocytochemistry, Gold probes, Silver enhancement, Transmission electron microscopy.

INTRODUCTION

During the development of immunocytochemical techniques, much effort has been directed toward labelling antigens while maintaining their structures the same as, or at least similar to the native state. One approach to this task involves the use of cryo-fixation and cryo-ultramicrotomy to minimize or eliminate denaturation of the antigens. However, the expense related to acquiring the necessary equipment limits the availability of this approach to many researchers. As an alternative, pre-embedding immunolabelling has obvious advantages over post-embedding immunolabelling because antibodies are introduced to the antigens before they are exposed to the harsh treatment of tissue processing.

Due to the large size of commercially available colloidal gold probes, pre-embedding immunocytochemical labelling at the ultrastructural level has mainly relied on other markers such as horseradish peroxidase (HRP). The recent introduction of one nanometer colloidal gold probes represents a significant advance in that the smaller size of the gold particle enhances probe penetration while maintaining cell or tissue morphology, and the decreased surface charge on the smaller gold particles facilitates the antigen-probe interaction. Visualization of small gold particles in biological systems has been achieved by increasing their size in a gold-catalyzed silver reduction procedure (1). The most recent versions are commercially available silver enhancement kits (Amersham, BioCell, and Aurion) that are more practical to use than original methods. The experimental applications of one nanometer gold probes and silver enhancement techniques have been reported in many studies (2,3,4). In this report, three experiments using this technique are described and the significance of this new technique for immunocytochemical localization in various types of cell and tissue preparations is discussed.

Experiment 1. *Tetrahymena pyriformis* is a ciliated protozoan with a complex cytoskeletal system. An important element of this cytoskeletal system is a microtubule associated filament 2-5 nm in diameter. Four subunit proteins of these filaments, called tetrins, have been isolated and characterized. They are arranged in different structures described as cages, cables, and networks of the cytoskeletal framework within the feeding organelle system, called the oral apparatus (5). In this experiment, a number of monoclonal antibodies specific for individual tetrin polypeptides were used to map their locations and determine whether the different packing arragements correlate with different distributions of the individual tetrin polypeptides. In order to free the filamentous structures from the cytoplasmic matrix for easy observation, *Tetrahymena* cells in this experiment needed to be extracted which also benefits the penetration of the immuno-reagents.

Experiment 2. Human alveolar macrophages (HAMs) produce a large amount of prostagladin E_2 (PGE_2), a potent anti-inflammatory mediator, following stimulation with lipolysaccharide (LPS) which is a substance present in the outer membrane of gram-negative bacteria. This process is in part dependent on new synthesis of the first committed enzyme, prostaglandin H (PGH) synthase. The localization of PGH synthase has been studied in mouse fibroblasts (6). The goal of this experiment was to determine if the newly synthesized PGH synthase is compartmentalized to a specific cellular structure in HAMs. Because of the importance of the membrane in protein synthesis, it is critical that the ultrastructure of the cells in this experiment be maintained.

Experiment 3. The nucleus tractus solitarius (NTS) is a structure in the dorsal medulla that receives visceral afferents (7). In rat brain, pharmacological and physiological studies suggest that glycine acts on cholinergic interneurons in the NTS and that this interaction may play an important role in the integration and mediation of cardiovascular reflexes and perhaps in the development of hypertension (8). To determine the relationship between glycinergic terminals and cholinergic neurons, double immunocytochemical labelling of glycine receptors (GlyR) using the avidin-biotin-peroxidase system (ABC kit) and choline acetyl transferase (ChAT) using one nanometer gold probes was performed. One unique feature of brain tissue is that it is filled with membrane bound cellular profiles and subcellular organelles. Therefore, the quality of membrane preservation is essential for analyzing cellular relationships in brain tissue.

The preceeding experiments using highly extrated cell suspensions, cell cultures and tissue sections are evaluated in regard to penetration of one nanometer colloidal gold probes, quality of ultrastructure, and sensitivity of the probes.

MATERIAL AND METHODS

Antibodies

Mouse monoclonal antibodies for tetrin polypeptides were provided by Dr. Norman Williams (9). Polyclonal rabbit antisera to sheep PGH synthase were raised by Dr. Robert Pueringer (10). Rabbit anti-GlyR antibody was purchased from Boehringer Mannheim (9115 Hague Rd., Indianapolis, IN. U.S.A.). Rabbit antiserum against ChAT was kindly provided by Dr. Louis B. Hersh at the University of Texas. One nanometer (Auroprobe One) and ten nanometer colloidal gold conjugated secondary antibodies were obtained through Amersham International (2636 S. Clearbrook Dr., Arlington Heights, IL. U.S.A.). ABC Elite kits, including biotinylated secondary antibodies and avidin-biotin-peroxidase complex were obtained from Vector Laboratories Inc (30 Ingold Rd., Burlingame, CA. U.S.A.).

One nanometer colloidal gold conjugated secondary antibodies can also be obtained from Biocell (Cardiff Business Technology Centre, Senghenydd Road, Cardiff CF2 4AY, UK) and Aurion (Vadaring 62, 6702 EA Wageningen, Netherlands).

Samples and fixations

Tetrahymena cells cultured in Williams' laboratory were extracted for 5 minutes in a PHEM buffer (11) solution (pH 6.9) containing 0.25% Triton X-100. The cells were fixed with 1% paraformaldehyde in the same extraction soluction for 30 minutes, and then transferred to 4% paraformaldehyde in phosphate buffer (pH 7.4) and fixed overnight. Human alveolar macrophages were isolated from normal volunteers and plated onto glass coverslips (10). The synthesis of PGH synthase was then stimulated by LPS (10 µg/ml) in RPMI-1640 tissue culture medium for 24 hours. Fixation was for at least 4 hours with paraformaldehyde-lysine-periodate (PLP) fixative (12) containing 2%

paraformaldehyde, 75mM lysine-HCL, 10mM sodium periodate and 37 mM phosphate buffer (pH 7.3) at 4°C. The cells were permeabilized with 0.05% Triton X-100 in phosphate buffered saline for 10 minutes prior to the immunogold labelling procedure. Male Sprague Dawley rats (225-250g) received an overdose of Nembutal (25mg) and were perfused intracardially with 0.9% NaCl followed by cold 4% paraformaldehyde and 15% saturated picric acid in phosphate buffer (pH 7.2). The brains were then dissected out and immersed in the same fixative for 1 hour. 50µm vibratome sections were cut from the medulla and permeabilized with 0.05% Triton X-100 in the blocking solution (see below).

Immunogold-labelling with one nanometer gold/silver enhancement

For labelling tetrins and PGH synthase, *Tetrahymena* and HAMs were washed after permeabilization and treated for 30 minutes with phosphate buffered saline (PBS) containing 5% normal goat serum, 0.8% bovine serum albumin and 0.1% cold water fish gelatin (blocking solution) to block non-specific labelling. Primary antibody incubations were for 2-3 hours at room temperature at an antibody dilution of 1:5 for mouse anti-tetrins or 1:500 for rabbit anti-PGH synthase. After 6 washes, cells were incubated for 3 hours with goat anti-mouse or goat anti-rabbit Auroprobe One diluted 1:50. For comparison, ten nanometer colloidal gold conjugated secondary probes were sometimes used with a dilution of 1:20. The cells were washed again and post-fixed with 2.5% glutaraldehyde for 15 minutes followed by 1% osmium tetroxide for 5 minutes. Silver enhancement of the colloidal gold was carried out by placing cells in Danscher's enhancement solution (1) for 15 (transmission electron microscopy) or 45 minutes (light microscopy) in the dark at room temperature. Details of the silver enhancement protocol are illustrated in Table 1. Following a thorough wash with distilled water, the cells were dehydrated and embedded in epoxy resins in the usual manner.

Double immunolabelling with one nanometer gold/silver enhancement and ABC kit.

For localizing glycine receptors and choline acetyl transferase in rat nucleus tractus solitarius, brain sections were incubated with blocking solution and then incubated overnight in a primary antibody solution containing a mixture of mouse anti-GlyR at 1:250 and rabbit anti-ChAT at 1:1000 dilutions. The secondary antibody solution was a mixture of biotinylated horse anti-mouse IgG (1:200) and goat anti-rabbit conjugated to one nanometer gold (1:50). Finally, sections were incubated in avidin-biotin-peroxidase solution as suggested by the manufacturer. After washes, the peroxidase enzyme reaction was carried out by incubating sections in a solution containing 0.1% of 3,3'-diaminobenzidine (DAB) and 0.0006% hydrogen peroxide for 20 minutes. Two different silver enhancement procedures, IntenSE M kit (Amersham) and Danscher's (1) were used to intensify gold labelling of ChAT according to the protocols described in Table 1. After dehydration, the sections were flat-embedded in Eponate 12 (Ted Pella, Inc, Redding, California, U.S.A.), and ultrathin sections were cut from the outer 5-10 µm of the vibratome sections.

TABLE 1. Silver enhancement protocols

IntenSE M	Danscher's
1. Post-fixation with glutaraldehyde 2.5%, 15 minutes	1. Post-fixation with glutaraldehyde 2.5%, 15 minutes
2. Wash with distilled water 3x3 minutes	2. Wash with phosphate buffer 0.1 M, 2x5 minutes
3. IntenSE M enhancement solution[1] 10 minutes	3. Post-fixation with OsO_4 1%, 10 minutes
4. Wash with distilled water 3x3 minutes	4. Wash with distilled water 5x3 minutes
5. Post-fixation with OsO_4 1%, 1hr	5. Danscher's enhancement solutión[2] 5 minutes
6. Wash with distilled water thoroughly	6. Wash with distilled water thoroughly

1. See manufacturer's instructions (Amersham)
2. See reference 1.

RESULTS

General

In the oral apparatus of *Tetrahymena*, tetrin-containing filaments were found to be associated with basal bodies of the oral cilia and microtubular bundles called cross-connectives (Figs. 1 and 2). Identical distributions were demonstrated with monoclonal antibodies directed against all four tetrins. In human alveolar macrophages, large amounts of PGH synthase were detected on the nuclear envelope of HAMs 24 hours after LPS stimulation (Fig. 3) In contrast, no PGH synthase was detected in the absence of stimulation. Choline acetyl-transferase immunoreactive dendrites and somata in the nucleus tractus solitarious were labelled with gold-silver particles. The post synaptic membranes of these structures were frequently labelled with DAB precipitate, signifying the presence of glycine receptors (Fig. 4).

The quality of ultrastructure and the intensity of gold-silver labelling

The ultrastructure in the tetrin mapping experiment was quite adequate for observing filaments even though the cells were highly extracted. The localization of tetrins in the oral apparatus was very consistent with both one nanometer and ten nanometer gold probes.

In the PGH synthase localization experiment, different amounts of gold labelling were observed in cells on the same section and the amount of labelling was always inversely related to the quality of the ultrastructure. It was also noted that when the nucleus was not located near the center of the cell, the amount of labelling in the region of the nuclear envelope closest to the cytoplasmic membrane (0.5-1 μm) was always higher. When satisfactory labelling was achieved on the side of the nuclear envelope further away from the cytoplasmic membrane (2-3 μm), some subcellular membrane structures, such as the endoplasmic reticulum and golgi apparatus, were often disrupted.

In rat brain vibratome sections, immunocytochemically labelled tissue could be divided into three zones. Both gold-silver particles and DAB precipitate were abundant in the superficial 1-2 μm zone where the morphology suffered from vibratome sectioning and detergent permeabilization. The amount of gold-silver particles decreased 2-3 μm below the superficial zone. However morphology was better as indicated by intact synaptic vesicle membranes, clearly visible neurotubules, uniform cytoplasm, and recognizable synaptic shapes. Most photographs were take from this zone. 2-3 μm below this intermediate zone, gold-silver particles disappeared while HRP labelled glycine receptors were still visible.

Comparison of one nanometer and 10 nm gold probes

When one nanometer gold probes were replaced by ten nanometer gold probes to label rabbit anti-PGH synthase antiserum in HAMs under the same permeabilizing conditions, no labelling was found on the nuclear envelope. When one nanometer gold probes were replaced with ten nanometer gold probes to label mouse anti-tetrin IgG in the oral apparatus of *Tetrahymena*, labelling was found in the same locations, but fewer particles were observed (Fig. 2).

Silver enhancement and post-fixation with osmiun tetroxide

Two silver enhancement procedures, IntenSE M and Danscher's, were compared in each experiment. In general, Danscher's procedure provides smaller and more evenly sized particles and therefore it was chosen for EM preparations. In Danscher's procedure, the size of silver particles produced is closely related to the incubation time and temperature of the silver solutions. In addition, the size of silver particles is also sample dependent. At room temperature, 10 minutes enhancement gave a particle size of 20-25 nm in *Tetrahymena* and HAMs, but about 100 nm in brain sections. It is also noticed that silver products from the two enhancement procedures responded to post-fixation with osmium tetroxide differently. There was no obvious loss of silver products after post-fixation with osmiun tetroxide when IntenSE M was used, therefore silver enhancement could be performed either before or after osmium fixation. However, when Danscher's procedure was used, silver enhancement had to be conducted after osmium fixation, otherwise almost all silver products would be lost.

DISCUSSION

The present studies demonstrate the utility of one nanometer colloidal gold conjugated probes and silver enhancement in pre-embedding immunolabelling in there common tissue preparations: highly extrated cells, intact cells in culture, and vibratome tissue sections. Several

aspects of this technique are evaluated with respect to each preparation.

Ultrastructure

In pre-embedding immunolabelling, the quality of ultrastucture is related to fixation, permeabilization, duration of antibody incubation, and in this study, silver enhancement. Glutaraldehyde fixation has been used with one nanometer gold immunolabelling (2), but more intense permeabilization is often needed because the stronger cross-linking of cytoplasmic proteins by glutaraldehyde blocks the penetration of the immuno-reagents. The consequence is that the positive effect of glutaraldehyde on morphology is offset by harsh permeabilization. Another concern about using glutaraldehyde is that the structure of the antigen is more likely to be changed. Thus, the aldehyde used in these studies was paraformaldehyde. However, the ultrastructure of a weakly fixed sample can deteriorate over long processing times. To reduce incubation times, two primary antibodies (from different species) were mixed together when double labelling was performed on brain tissue.

The silver enhancement solution has a low pH which can also damage ultrastructure. Therefore, silver enhancement after fixation with osmium tetroxide or glutaraldehyde is advantageous. It should be noted that it is difficult to monitor the progress of the silver reaction after osmification because the tissue is already darkened by the osmium.

Penetration

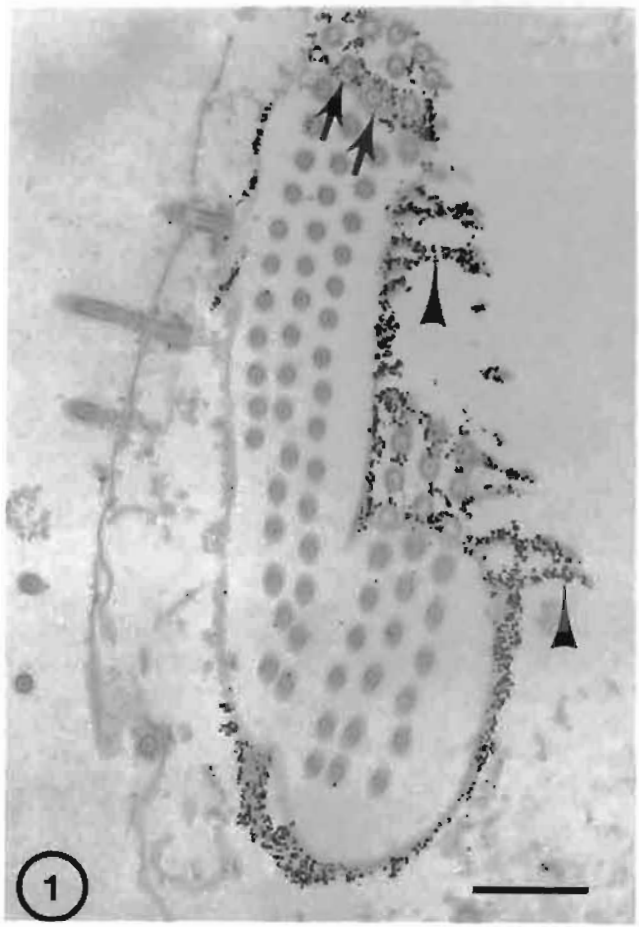
The greater penetration of the one nanometer gold probes is especially beneficial for the intracellular labelling on the surface of vibratome tissue sections where all subcellular profiles are exposed to the immuno-reagents. In this study, brain sectins were permeabilized with Triton X-100 to improve the penetration, but excellent labelling has also been reported with less detergent or in the absence of detergent treatment (13). In intact cells (HAM's), subcellular structures are not only surrounded by the cell membrane, but also immersed in the cytoplasmic matrix. Although the one nanometer gold probe provedes better penetration then the larger gold probes, cells still need to be permeabilized, often to the degree that subcellular structures are difficult to recognize. Penetration of the avidin-biotin-HRP probe in HAMs was not tested in this study, but the results from the double labelling of glycine receptors and choline acetyl-transferase in brain tissue demonstrated that the avidin-biotin-HRP probe penetrates to a greater depth which suggests that better results may be achieved if the avidin-biotin-HRP probe is used in intact cell. Compared to the avidin-biotin-HRP probe, the size of a one nanometer gold probe is presumably smaller. It consists of an immunoglobulin with a molecular weight of 150 KD and 1-3 gold particles of 30 atoms. However, since gold particles carry negative surface charges, a water shell is often formed which results in an increase in the actual size of the probes.

Triton X-100 is a common detergent used for permeabilizing cell membranes. It is a small, nonionic amphipathic molecule that tends to form micelles in water. The hydrophobic ends of the detergent molecules disrupt the lipid bilayer and bind to the hydrophobic regions of the membrane proteins. Since the other end of the detergent molecule is polar, this binding tends to pull the membrane proteins into solution as detergent-protein complexes, thus leaving cell membranes with holes. It should be emphasized that detergent concentration and duration of permeabilization is sample dependent. A series of variations should be tested when different types of samples are investigated.

Sensitivity

In this study, *Tetrahymena* cells were highly extracted, therefore ten nanometer gold particles were able to penetrate the full extent of the cells. Thus, the use of one nanometer gold particles did not offer any advantage in this respect. However, stronger signal was detected when one nanometer gold probe was used, suggesting that this probe permits greater sensitivity than is possible with ten nanometer particles. This is presumably due to the lower negative surface charge of the smaller gold particles which results in less non-specific repulsion between the probe and the sample than occurs when larger probes are used.

In conclusion, the use of one nanometer colloidal gold conjugated antibody probes combined with silver enhancement is a valuable advance in pre-embedding immunolabelling. It not only offers an alternative marker system, but also makes it possible to do preembedding double immunolabelling. However, this approach is not necessarily suitable for all systems, especially if cell morphology is seriously compromised by detergent permeabilization and prolonged treatment during the immuno-labelling procedure. Given the wide acceptance of immuno-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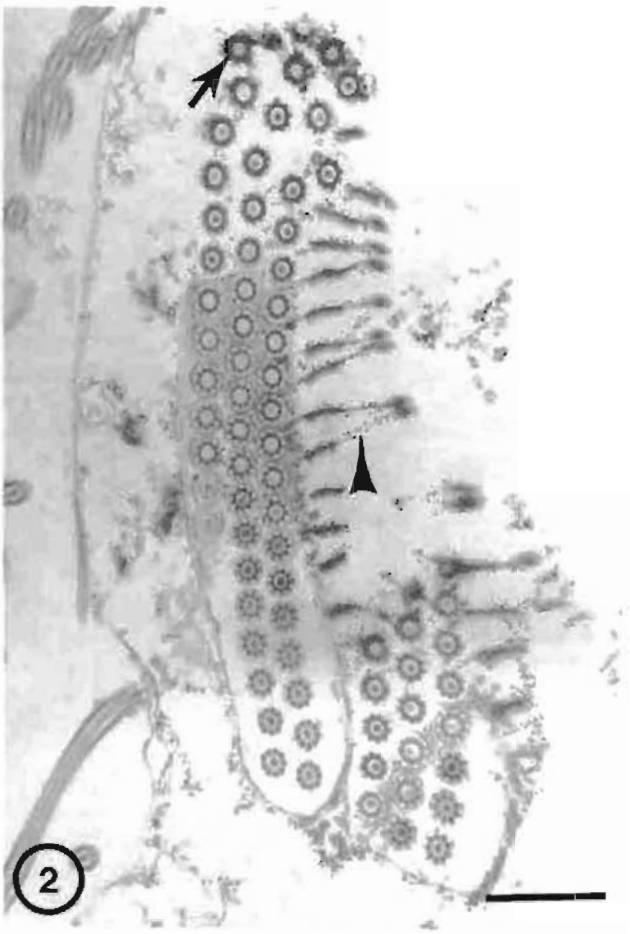
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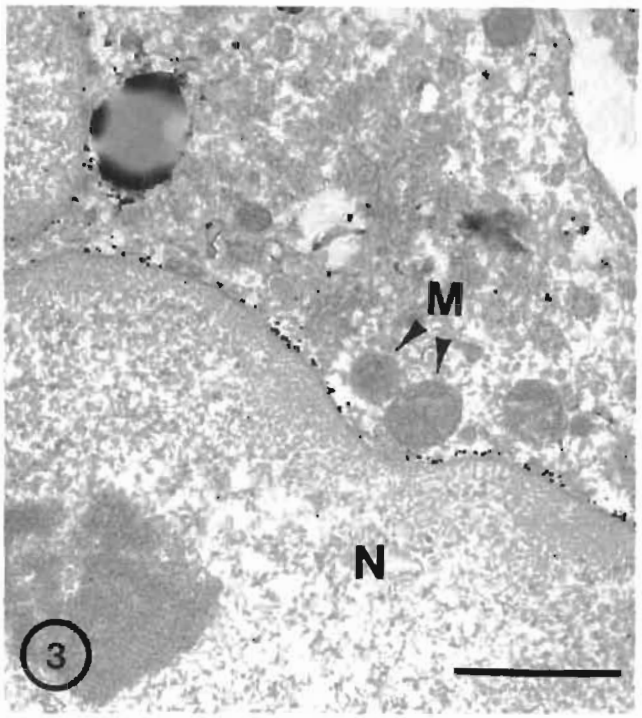
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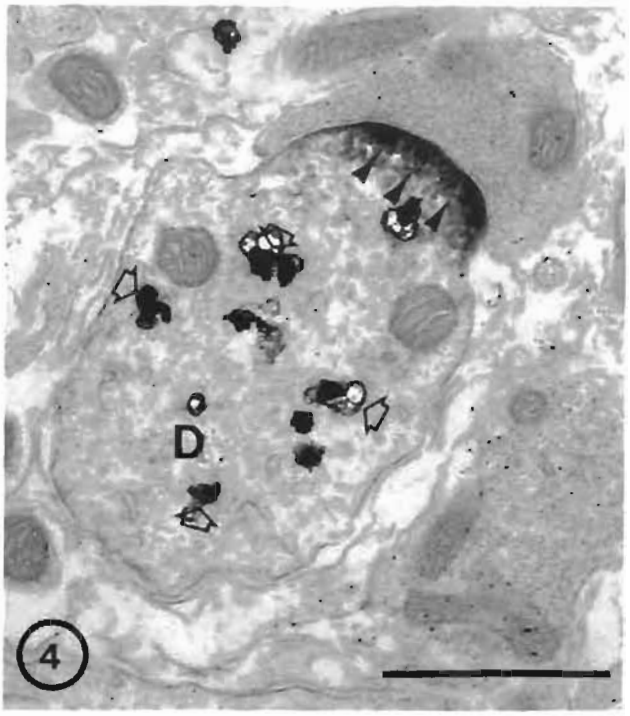
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electron microscopy, investigators need to be aware of the different methods available for handling specimens. In future papers, the use of post-embedding labelling including cryo-ultramicrotomy and resin embedded samples will be discussed using practical results from experiments done in the University of Iowa Central Electron Microscopy Research Facility.

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RESUMEN

Como un procedimiento alternativo al inmunomarcaje post-embebimiento de las muestras en resina, el inmunomarcaje pre-embebimiento, incrementa la probabilidad del reconocimiento de antigenos por los anticuerpos durante la localización inmunocitoquímica. El desarrollo de anticuerpos conjugados a oro coloidal de un nanometro ofrece nuevas sondas para el inmunomarcaje intracelular en el pre-embebimiento. En este trabajo, el uso de sondas de oro coloidal de un nanometro resaltadas con plata acoplada, fue examinado en tres muestras biológicas comúnmente usadas: suspensiones celulares extraidas, cultivos celulares permeabilizados por detergentes y secciones de tejido obtenidas por vibratomo. Las sondas de oro coloidal de un nanometro demostraron una mayor sensibilidad que las sondas de oro coloidal más grandes, sobre preparaciones celulares extraídas. Sin embargo, células intactas necesitan ser drásticamente permeabilizadas para permitir que las sondas de oro de un nanometro alcancen los sitios antigénicos. Sondas de oro de un nanometro pueden penetrar 4 - 5 μ en el interior de secciones de vibratomo permeabilizadas con detergente.

REFERENCES

1. Danscher, G. (1981) Localization of gold in biological tissue. A photochemical method for light and electron microscopy. Histochemistry. **71**: 81-92

2. De Graaf, A., Van Bergen En Henegouwen, P.M.P., Meijne, A.M.L., Van Driel, R. and Verkleij, A.J. (1991) Ultrastructural localization of nuclear matrix proteins in Hela cells using silver-enhanced ultra-small gold probes. J. Histrochem. Cytochem. **39**: 1035-1045

3. Farrel, C.D. and Pardridge W.M. (1991) Utrastructural localization of blood-brain barrier-specific antibodies using immunogold-silver enhancement techniques. J. Neurosci-Methods **37**: 103-110

4. Kato , D., Yokose S. and Tajima, Y. (1991) Use of silver enhancement technique for immunohistochemical detection of epidermal growth factor in rat submandibulargland. Biotech. Histochem. **1**: 87-88

5. Williams, N.E., (1986) The nature and organization of filaments in the oral apparatus of

Figs. 1-2 Distribution of tetrin polipeptides in the oral apparatus of Tetrahymena. Immunolabelling is found to be associated with the basal bodies of the oral cilia (arrows) and microtubular bundles (arrowheads). Tetrahymena in suspension were highly extracted prior to the application of antibodies. Note that the labelling is more intense on Fig. 1.

Fig. 1. Immunolabelling of tetrin using one nanometer colloidal gold conjugated probes coupled with silver enhancement (Danscher's procedure). Bar = 1mm.

Fig. 2. Immunolabelling of tetrin using ten nanometer colloidal gold conjugated probes (no silver enhancement). Bar = 1mm.

Fig. 3 Immuno-localization of prostaglandin H synthase in human alveolar macrophages 24 hours after lipopolysaccharide stimulation. The cells were permeabilized with 0.05% Triton X-100 for 10 minutes prior to the application of the antibodies. One nanometer colloidal particles were silver enhanced using Danscher's procedure. M, mitochondria; N, nucleus. Bar = 1mm.

Fig. 4 Double immuno-locatization of glycine receptors and choline acetyl-transferase in the nucleus tractus solitarious of the rat brain. The profile of a choline acetyl-transferase immunoreactive dendrite (D) is labelled with gold-silver particles (arrows). The post synaptic membrane of this dendrite is labelled with DAB precipitate (arrowheads), indicating the presence of glycine receptors. Brain vibratome sections were peameabilized with 0.05% Triton X-100 for 30 minutes prior to the application of the antibodies. Bar = 1mm.

Tetrahymena. J. Protozoal., 33: 352-358
6. Rollins, T.E. and Smith, W.L., (1980) Sub-cellular localization of prostaglandin forming in Swiss mouse 3T3 fibroblast by electron microscopic immunocytochemistry. J. Biol. Chem. 255: 4872-4875
7. Torvik, A. (1956) Afferent connections to the sensory trigeminal nuclei, the nucleus of the solitary tract and adjacent structures. An experimental study in the rat. J. Comp. Neurol. 106: 51-141
8. Talmal, W.T., Kauth-Colling, J.M. and Robertson, S.C. (1991) Glycine microinjected into nucleus tractus solitari of rat acts through cholinergic mechanisms. Am. J. Physiol. 260: H1326-H1331
9. Dress, V,M., Yi, H., Musal, M.R. and Williams, N.E., (1992) Tetrin polypeptides are co-localized in the cortex of *Tetrahymena*. J. Structural Biol., 108: 187-194
10. Pueringer, R. J. and Hunninghake, G. W.,(1992) Ameri. J. Physiol., Lipopolysaccharide stimulates de novo synthesis of PGH synthase in Human alveolar macrophages. 261: L78-L85
11. Schliwa, M. and Blerkon, J. V., (1981) Structural interactions of cytoskeletal components. J. Cell Biol., 90: 222-235
12. Mclean, I. W. and Nakane P. K., (1974) Periodate-lysine-paraformaldehyde fixative. A new fixative for immunoelectron microscopy. J. Histochem. Cytochem., 22 (12): 1077-1083
13 Chan, J., Aoki, C. and Pickel, V. M., (1990) Optimization of differential immunogold-silver and peroxidase labelling with maintenance of ultrastructure in brain sections before plastic embedding. J. Neurosci. Methods. 33: 113-127