

# Immunoperoxidase Technique in Cryoultramicrotomy for the Detection of Simian Adenovirus in Cell Nuclei

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## INTRODUCTION

The application of immunological concepts in morphological studies is in rapid progress. While many cell components can be studied by pre- or postembedding immunohistology, one would like to possess an alternative method in order to overcome all problems observed in pre- and postembedding staining due to penetration difficulties e.g. for antibodies and cell alterations by dehydration and resin curing (4). Thus, the development of cryoultramicrotomy (1-2, 12) offered great promise for immuno-staining of cells to be done on ultrathin frozen sections without classical procedures of dehydration and plastic embedding. Several immunohistological procedures including various marker systems were tried with ultrathin frozen sections, but until now reasonable results were obtained only with ferritin as label (13). Hitherto, direct or indirect peroxidase stainings were most often disappointing inasmuch as the original 3,3'-diaminobenzidine method (3) was very capricious when applied to ultrathin frozen sections (4, 7). Recently, an approach was published for the immunoperoxidase localization of hormones in pituitary gland by use of 4-chloro-1-naphthol as substrate (9). However, the authors' tissue processing *per se* (glutaraldehyde fixation followed by postfixation with osmium tetroxide and uranium salt staining prior to immuno-staining) leads already to such a strong contrast of cellular organelles that the immunohistological reaction is hard to be discerned.

Because of the general interest in the peroxidase labelling method in cell biology we searched for further developments of this technique in cryoultramicrotomy. In this note we describe our recent progress. We have used simian adenovirus type 7 (SA<sub>7</sub>) infected monkey kidney cells in order to localize virus antigens in the cell nucleus. We are aware that the employed anti-human adeno 7 immune sera possessed antibody specificities against the bulk of virus antigens, but these immune sera are sufficient to demonstrate feasibility of the immunoperoxidase method in cryoultramicrotomy.

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## MATERIALS AND METHODS

Rabbit anti-human adeno type 7 immune sera were purchased from Microbiological Associates (Walkersville, U.S.A.). Nuclear SA<sub>7</sub> virus was stained by an indirect peroxidase labelled antibody method. For this purpose, an anti-rabbit IgG immune serum was produced in sheep from which specific anti-rabbit IgG antibodies were isolated and conjugated with horseradish peroxidase (HRP, RZ 3). Antibody-peroxidase conjugates were purified by gel filtration and affinity chromatography (4-5, 8). Immunohistological control sera included unlabelled and HRP-labelled normal rabbit IgG globulins and rabbit anti-glucose oxidase antibodies.

Experiments were done with cultivated kidney cells of *Cercopithecus aethiops* infected with 10 p.f.u./cell. Sixty-two hr after infection, cultures were fixed *in situ*. Formaldehyde (from paraformaldehyde) and glutaraldehyde (PI = 0.1-0.2) were used in 0.2 M cacodylate-HCl buffer pH 7.2. Fixations were carried out with 4% formaldehyde for 10 min followed by 4% formaldehyde plus 0.25% glutaraldehyde for 10 min at 0 °C. Then, cells were harvested from the plastic dish and centrifuged at 5,000 rpm for 5 min. The obtained pellets were washed in cacodylate buffer for 3-5 hr with frequent changes of the buffer solution.

Cell pellets were infiltrated with sucrose as described (12). Then preparations were frozen on a tissue holder by immersing into liquid nitrogen and placed into the cryochamber of a Sorvall MT-2 cryoultramicrotome; knife and trough at -70°C. Sections were spread on 50% dimethylsulfoxide (DMSO) in distilled water, transferred with a loop on a drop of water at room temperature, collected on Formvar (0.3%) and carbon-coated nickel grids and dried overnight at room temperature (11).

Prior to immunohistological reactions, grids were rehydrated for 1 min on a drop of 0.01 M phosphate buffered saline pH 7.2 (PBS) and passed on a drop of PBS supplemented with 3% sheep normal serum (PBS/sheep). Then grids were incubated with anti-adeno type 7 immunoglobulins (0.1 mg/ml) for 5 min and followed by HRP-labelled sheep anti-rabbit IgG antibodies (0.1 mg/ml) for 5 min; unreacted antibodies were washed off by three successive passages on droplets of PBS/sheep. After one rinse in 0.2 M Tris-HCl buffer pH 7.4, grids were held for 5 min with forceps into continuously stirred enzyme substrate (modified from Graham and Karnovsky, 1966) (3): 2.5 mg 3,3'-diaminobenzidine · 4 HCl (DAB) in 10 ml Tris-HCl buffer supplemented with 25 µl of 1% H<sub>2</sub>O<sub>2</sub>. Finally, grids were rinsed by three passages on PBS and one passage on distilled water, then air-dried and examined in an electron microscope operating at 80 kV with a 50 µm objective aperture.

## RESULTS AND DISCUSSION

### 1. Tissue stabilization

The first step in combined cryoultramicrotomy and immuno-staining was the adaptation of an appropriate fixation procedure by which stabilization of cell structure was achieved without significant loss of its immunoreactivity. In a set of experiments, schedules of different formaldehyde and glutaraldehyde concentrations and various fixation times

were examined on SA7 infected cultures in order to control both immunoreactivity and ultrastructural preservation. The above described fixation method proved satisfactory for our purpose. Prior to cryoultramicrotomy, pellets of the aldehyde-fixed cells were infused with sucrose. This step was important for our material because in contrast to solid tissue blocks the *in vitro* cultivated cells proved very fragile. Sucrose infusion enhances plasticity of cells and prevents freezing-damage by a too large ice crystal formation (12).

## **2. Cryoultramicrotomy**

Frozen cut sections were usually spread on DMSO and transferred to room temperature, but in few cases sections were also directly taken from the knife by a drop of sucrose solution and brought outside the cryochamber (12). The latter technique was more intricate, but was superior to DMSO spreading in the knife trough. However, at the early stages of our study we preferred the collection of sections on DMSO. This permitted us to prepare more rapidly the necessary numbers of grids for the diverse experiments and controls.

Overnight dried sections were found to stabilize further the ultrastructure of cells when we submitted grids either immediately or after overnight drying to immunohistology. Indeed, ultrastructure of overnight dried and then immuno-stained cells was reproducibly better conserved than that of the immediately immuno-stained preparations. The antigens studied by us were not denatured by air-drying.

## **3. Immunoperoxidase technique on ultrathin frozen sections**

Virus particles were readily revealed by immunoperoxidase reaction of ultrathin frozen sections in the nuclei of infected cells. The cytochemical reaction was strong and confined to the viruses which were specially arranged in a pseudocrystalline form in the inclusion bodies (Fig. 1, 2). This distribution corresponds to that which has been described by classical electron microscopy (10). Uninfected cells did not stain. Also, infected cells which were incubated with unrelated antibodies (anti-glucose oxidase) or with normal rabbit IgG did not stain (Fig. 3).

As a matter of fact, specificity of immuno-staining depends on the quality of immunological reagents. General aspects of preparation of immunohistological reagents have been given earlier (4). In the present study, interaction of tissue sections with non-antibody globulins, with unrelated antibodies and with HRP conjugates did not occur. Pretreatment of sections with non-immune serum of the same species in which the "sandwich" antibodies (sheep) were raised proved effective in prevention of nonspecific adsorptions. The employed concentrations of immunoglobulins and HRP conjugates were empirically determined by experiments at both light and electron microscopic levels.

We have noted in earlier publications difficulties in immunoperoxidase labelling of cell components in ultrathin frozen sections (4-6). These difficulties were attributed to the original DAB cytochemistry which gave heavy non-specific precipitations of oxidized DAB and which obscured cellular details. In subsequent studies, incubation times were drastically shortened and, moreover, grids were passaged on serial baths of freshly prepared enzyme substrate (7). This procedure enhanced staining specificity, but meaningful progress was not achieved until the schedules described here were developed.

Our results will contribute to the study of methods for the localization of immunoreactive ligands in intracellular spaces which are difficult to detect by pre- or post-embedding immunohistology. It is supposed that the described procedures can be adapted to other cell systems.

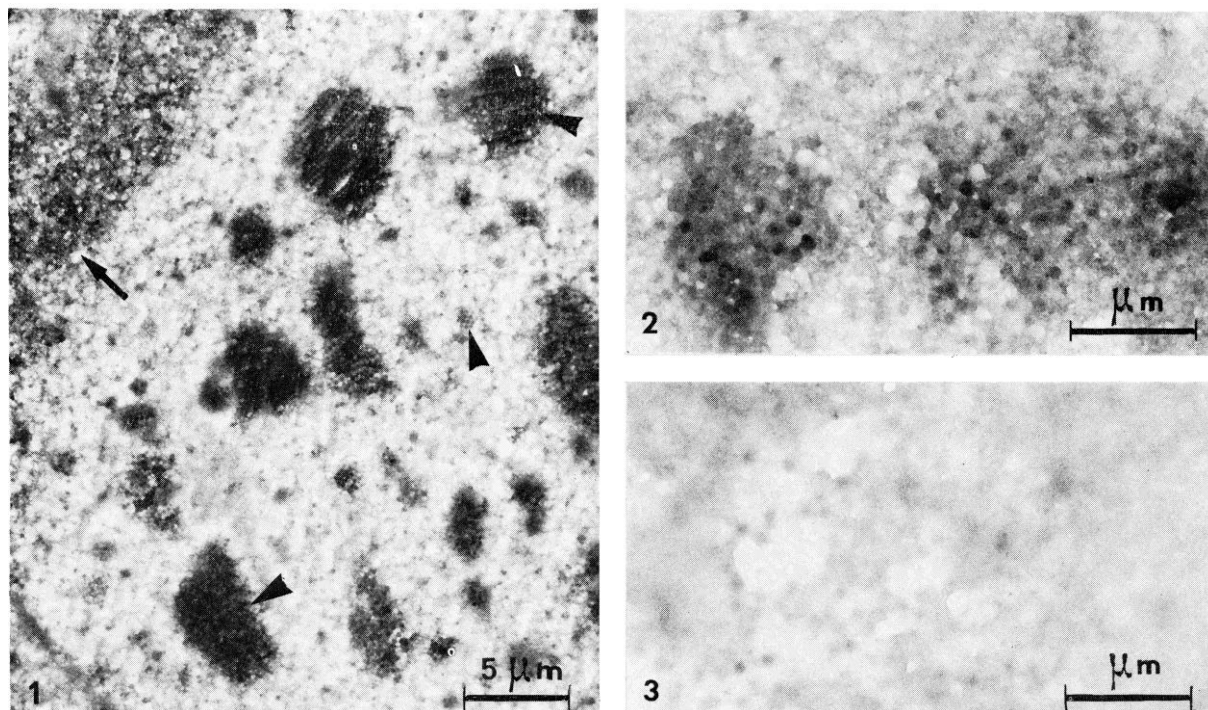


FIGURE 1. – Immunoperoxidase reacted SA<sub>7</sub> viruses in the nucleus of an infected cell. Note staining of dispersed virus particles at the periphery of the nucleus (←) and viruses in pseudo-crystalline form within the inclusion body (▶); x 3,000.

FIGURE 2. – High magnification view of immunoperoxidase stained viruses in the inclusion body; x 18,000.

FIGURE 3. – Infected cells, control reaction. No staining is observed after incubation with unrelated antibody, HRP conjugate and enzyme substrate; x 18,000.

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