

# Diaminobenzidine Cytochemistry in Cryo-Ultramicrotomy for the Detection of Peroxidases

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## Summary

Rat erythrocytes and lymphoid cells were fixed by glutaraldehyde and encapsulated in bovine serum albumin plus rabbit IgG globulins for cryo-ultramicrotomy. A technical procedure is described by which endogenous peroxidases of erythrocytes in ultrathin frozen sections were detected by hydrogen peroxide and diaminobenzidine as hydrogen donor. Modifications of this classical cytochemical procedure proved also useful in cryo-ultramicrotomy for immunoperoxidase labeling of antigenic determinants in rabbit IgG globulins which have been cross-linked within the supporting matrix of cells.

## Introduction

There has been a growing trend among cytologists toward cytochemical localization of specific constituents at the ultrastructural level. While many components can be identified in plastic embedded material, a newer approach which holds great promise is that involving the use of ultrathin frozen sections. Especially for immunocytochemical work, cryo-ultramicrotomy offers the definite advantage since penetration of labeled antibodies is thought to be no longer a problem. Developments of procedures for cryo-ultramicrotomy, suitable for cytochemical and immunocytochemical procedures have been published (Bernhard and Nancy, 1964; Dollhopf et al., 1969; Kolemäinen-Seveus, 1970; Bernhard and Viron, 1971; Iglesias et al., 1971; Kuhlmann and Viron, 1972; Painter et al., 1973; Tokuyasu and Singer, 1976). For the latter mostly ferritin labeling techniques were employed. The disadvantage of ferritin, however, is its often observed non specific sticking to tissue sections which could be overcome in some cases (Tokuyasu and Singer, 1976). Hence, peroxidase labeled antibodies are widely used either in preembedding or in postembedding cytology (for review see Kuhlmann, 1977) and its application to ultrathin frozen sections is expected to be useful, too.

The introduction of the diaminobenzidine (DAB) cytochemistry (Graham and Karnovsky, 1966) proved until now to be most useful for the ultrastructural detection of endogenous peroxidase activity in cells and also in ultrastructural immunoperoxidase labeling (Kuhlmann, 1977). However, this staining technique was still very capricious when applied to ultrathin frozen sections (Kuhlmann and Miller, 1971; Kuhlmann et al., 1974). Thus, it remained to be shown

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that the DAB technique might be successfully employed in cryo-ultramicrotomy and, especially, when immunoperoxidase labeling should be employed. In this communication we show that the detection of peroxidases by DAB can be reliably performed. For this purpose, prefixed erythrocytes which contain endogenous peroxidases and prefixed lymphocytes which do not contain stainable peroxidases (by this procedure) were encapsulated together in a mixture of bovine serum albumin plus rabbit IgG globulins and processed for frozen thin-sectioned cytochemistry. Intracellular peroxidases in erythrocytes as well as extracellular rabbit IgG were detected by reacting sections with enzyme substrate alone or with specific peroxidase conjugates followed by enzyme substrate.

## Materials and Methods

*Tissues.* Rat popliteal lymph nodes were taken, teased with forceps in phosphate buffered saline (PBS) and brought into suspension. Cell suspensions were washed two times in PBS by centrifugations at 100 x g for 5 min at 4° C, then fixed in ice-cold 1.5% glutaraldehyde/0.2 M cacodylate buffer pH 7.2 for 30 min. Rat erythrocytes were prepared by collection of heparinized blood which was added dropwise to ice-cold 1.5% glutaraldehyde and fixed for 30 min at 4° C. Fixation of cells was stopped by successive centrifugations at 100 x g/5 min in cacodylate buffer.

*Encapsulation of Cells.* Encapsulation of prefixed cells for cryo-ultramicrotomy was essentially as described (Kuhlmann and Viron, 1972). Briefly, a solution of 15% bovine serum albumin (BSA) was prepared in 0.2 M cacodylate buffer pH 7.2 and pure rabbit IgG globulins (Nordic Lab., Netherlands), 10 mg/ml, were added. Prefixed pellets of lymphoid cells and erythrocytes were suspended in encapsulation solution, mixed and centrifuged in a collodion bag (Sartorius, Germany) at approx 200 x g for 5 min. The collodion bag containing the cell pellet was transferred into a tube with 5% glutaraldehyde. Polymerization of cells with encapsulation medium occurred immediately by diffusion of glutaraldehyde across the membrane, then the latter was cut off and the insolubilized mixture was cut into cubes of approx 1 mm<sup>3</sup> (Fig. 1 inset). Blocks were washed at least overnight in several changes of buffer.

*Cryo-Ultramicrotomy.* Prior to freeze-sectioning, blocks were pretreated with 20% dimethyl sulfoxide (DMSO) buffered with cacodylate for 20 min. Preparations were frozen on a tissue holder by immersing both into liquid nitrogen-cooled isopentane. Sections were made with either the Sorvall cryokit MT 2 microtome (Sorvall, USA) or the LKB cryokit (LKB, Sweden). Sections were spread in the trough on 50% DMSO/cacodylate buffer, collected with Marinozzi rings and washed at laboratory temperature in cacodylate buffer.

*Cytochemical Procedures.* Sections were transferred on Formvar coated 200 mesh grids. Unless stated elsewhere, sections were reacted by negative staining with 4% sodium silicotungstate in water for 15 s at 37° C (Bernhard and Viron, 1971) (Fig. 1).

Endogenous peroxidases were stained by floating grids on droplets of the original DAB plus hydrogen peroxide substrate (Graham and Karnovsky, 1966) varying from 1 to 10 min at laboratory temperature and using 5 serial passages. Then, grids were rinsed for 2 min by serial passages on cacodylate buffer and either postfixed or not with different concentrations of OsO<sub>4</sub> (0.1-0.5% in cacodylate buffer) for 1 min, followed by washings

in distilled water. Controls were done with media in which  $\text{H}_2\text{O}_2$  was omitted. Inhibition of endogenous peroxidases was tested by floating grids on 0.5 to 1%  $\text{H}_2\text{O}_2$  for 5-15 min prior to incubation in the above substrate medium.

Extracellular rabbit IgG globulins within the encapsulation medium were stained after inhibition of endogenous peroxidases according to this schedule: (a) grids were floated on 0.5 mg BSA/ml cacodylate buffer during 5 min, followed by short wash in cacodylate buffer; (b) incubation on a droplet of peroxidase labeled sheep anti-rabbit IgG antibodies (Kuhlmann, 1975), 0.1 mg/ml for 5 min, followed by at least 5 serial passages on cacodylate buffer, postfixation with 0.5% glutaraldehyde for 1 min and 5 serial passages on cacodylate buffer; (c) peroxidase activity was revealed by the procedure described above.

Grids were allowed to dry and examined in a Siemens Elmiskop IA operating at 80 kV and a 50  $\mu$  objective aperture.

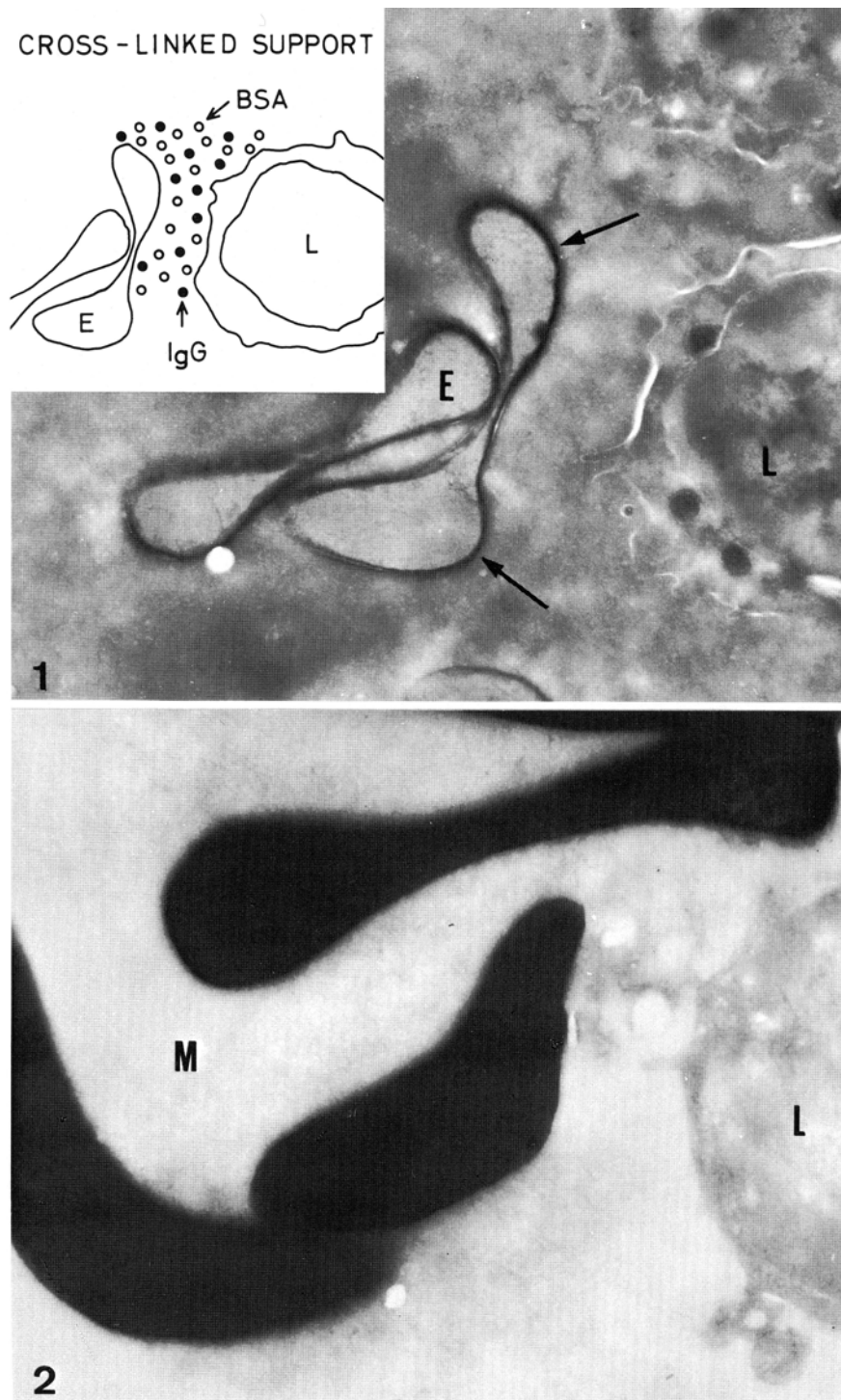
## Results and Discussion

The proposition of cryo-ultramicrotomy enlarges the number of choices available to investigations of ultrastructural cytochemistry. Several attempts have been made to combine this technique with cytochemistry since 1964 (Bernhard and Nancy, 1964; Iglesias et al., 1971; Bernhard and Viron, 1971; Kuhlmann and Viron, 1972; Tokuyasu, 1973) as well as with immunoperoxidase labeling (Kuhlmann and Miller, 1971; Kuhlmann et al., 1974; Kuhlmann, 1977) both for the ultrastructural localization of cellular components. In the latter technique, however, the main difficulty encountered in DAB cytochemistry was the observation that either oxidation of DAB or its accumulation at the enzyme site did not occur in the usual manner. The purpose of the present communication is to show now the feasibility of DAB cytochemistry for visualization of peroxidase activities in ultrathin frozen sections. Details for the use of cross-linked proteins as encapsulating support in cryo-ultramicrotomy, the preparation of ultrathin frozen sections and of specific immunocytochemical reagents were described elsewhere (Kuhlmann and Viron, 1972; Kuhlmann, 1977).

*Detection of Endogenous Peroxidases in Cells.* Incubation of ultrathin frozen sections with the original DAB/ $\text{H}_2\text{O}_2$  substrate mixture during the described reaction time (Graham and Karnovsky, 1966) usually resulted in heavy non specific precipitations and artifactual diffusion of oxidized diaminobenzidine which obscured cellular details. Upon simple postfixation of sections with buffered osmium tetroxide in usual concentrations (higher than 0.5%), also non specific precipitates concomitant with damage to cytoplasmic and nuclear structures were observed.

Optimal and distinct staining of endogenous peroxidases in erythrocytes were obtained when sections were incubated on substrate medium for not longer than 5 to 6 min. During this period, grids were passaged on freshly prepared substrate droplets for at least 5 times. After rinsing with cacodylate buffer, postfixation with osmium was not necessary but enhanced much more electron density of the reaction product. Good results were obtained with  $\text{OsO}_4$  concentrations not higher than 0.2% for 1 min (Fig. 2). Large and small lymphocytes as well as plasma cells and the encapsulating medium did not stain by the enzyme substrate. Control sections in which  $\text{H}_2\text{O}_2$  was omitted, erythrocytes also did not stain. When sections were previously incubated in 0.5

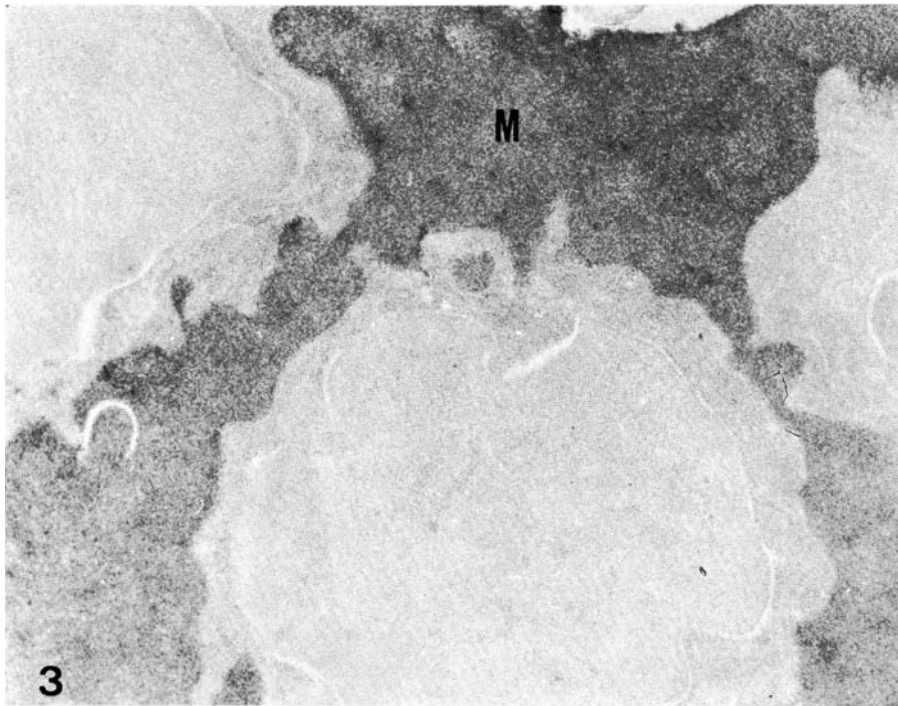
to 1%  $\text{H}_2\text{O}_2$  for at least 5 min, endogenous peroxidases could be no longer detected by the above procedure.



**Fig. 1.** Negative stain of section from encapsulated erythrocytes (E) and lymphoid cells (L). The reason for the prominent appearance of erythrocyte membranes and cell-coats ( $\rightarrow$ ) is not known,  $\times 10,000$ . *Inset:* schematic drawing of sectioned material; note erythrocytes (E) and lymphoid cell (L) supported in encapsulation matrix which consists of insolubilized BSA (o) and rabbit IgG globulins ( $\bullet$ )

**Fig. 2.** Detection of endogenous peroxidases in erythrocytes. Note unstained lymphoid cell (L) and encapsulation matrix (M)  $\times 15,000$

*Visualization of IgG Globulins by Peroxidase Labeled Antibodies.* Since encapsulated cell preparations proved useful for the detection of endogenous peroxidases by the described procedure, our aim was to use this staining technique also for immunocytochemical purposes in which horseradish peroxidase was coupled as a marker to specific antibodies. Antigenic determinants of rabbit IgG globulins which were incorporated in encapsulating medium and subsequently cross-linked together with glutaraldehyde could be specifically stained by peroxidase conjugates of sheep anti-rabbit IgG antibodies; lymphoid cells did not stain as expected (Fig. 3).



**Fig. 3.** Visualization of rabbit IgG globulins by peroxidase labeled sheep anti-rabbit IgG antibodies and followed by enzyme substrate. Note high electron density of encapsulation matrix (M) and clear appearance of cells; section was not postfixed with OsO<sub>4</sub>, x 15,000

The advantage of diaminobenzidine in cytochemistry is the high insolubility of the oxidized product and its osmiophilic character, thus giving excellent electron density. The described modification of DAB staining procedure could be employed equally well for the detection of endogenous peroxidases, f.e. in erythrocytes, and for immunoperoxidase labeling in ultrathin frozen sections. Important points in this technique were to use short incubation times throughout and in the case of postosmication low concentrations, too. Finally, it must be strengthened that in combining cryo-ultramicrotomy and cytochemistry in which incubations are to be performed just in aqueous solutions, the use of well "insolubilized" components (by fixation, cross-linkage) are recommended. Otherwise, diffusion artifacts must be expected (Kuhlmann et al., 1974; Kuhlmann, 1977).

In previous immunocytochemical studies on the ultrastructural localization of either anti-enzyme antibodies or IgG globulins in lymphoid cells by immunoperoxidase labeling (Kuhlmann and Miller, 1971; Kuhlmann et al., 1974), we have shown that appropriate fixation procedures must be elaborated independently for each biological material. Especially, glutaraldehyde is an excellent cross-linking agent which was introduced by Sabatini et al. (1963) for ultrastructural

work, but it must be kept in mind that this fixative may hinder the accessibility of antigenic determinants by labeled antibodies. Consequently, the effect of aldehyde fixation on the immunological reactivity of antigens must be examined and reconciled with conservation of cellular ultrastructure. These two goals are often contradictory and cannot always be obtained simultaneously.

## References

- Bernhard, W., Nancy, M.T.: Coupes à congélation ultrafines de tissu inclus dans la gélatine. *J. Microscop.* **3**, 579-588 (1964)
- Bernhard, W., Viron, A.: Improved techniques for the preparation of ultrathin frozen sections. *J. Cell Biol.* **49**, 731-746 (1971)
- Dollhopf, F.L., Werner, G., Morgenstern, E.: Die Shandon-Reichert-Kühleinrichtung zum Herstellen von Ultradünn- und Feinschnitten bei extrem niederen Temperaturen. II. Anwendungsbeispiele aus Biologie und Technik. *Mikroskopie* **25**, 33-47 (1969)
- Graham, R.C., Karnovsky, M.J.: The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique. *J. Histochem. Cytochem.* **14**, 291-302 (1966)
- Iglesias, J.R., Bernier, R., Simard, R.: Ultracryotomy: a routine procedure. *J. Ultrastruct. Res.* **36**, 271-289 (1971)
- Kolemainen-Seveus, L.: Frozen ultrathin sections. *Proc. 7th Int. Congr. Electron Microsc.* **1**, 432-433 (1970)
- Kuhlmann, W.D.: Purification of mouse alpha<sub>1</sub>-fetoprotein and preparation of specific peroxidase conjugates for its cellular localization. *Histochemistry* **44**, 155-167 (1975)
- Kuhlmann, W.D.: Ultrastructural immunoperoxidase cytochemistry. *Progr. Histochem. Cytochem.* **10**, 1-57 (1977)
- Kuhlmann, W.D., Avrameas, S., Ternynck, T.: A comparative study for ultrastructural localization of intracellular immunoglobulins using peroxidase conjugates. *J. Immunol. Meth.* **5**, 33-48 (1974)
- Kuhlmann, W.D., Miller, H.R.P.: A comparative study of the techniques for ultrastructural localization of antigen-antibody complexes. *J. Ultrastruct. Res.* **35**, 370-385 (1971)
- Kuhlmann, W.D., Viron, A.: Cross-linked albumin as supporting matrix in ultrathin cryo microtomy. *J. Ultrastruct. Res.* **41**, 385-394 (1972)
- Painter, R.G., Tokuyasu, K.T., Singer, S. J.: Immunoferritin localization of intracellular antigens: the use of ultracryotomy to obtain ultrathin sections suitable for direct immunoferritin staining. *Proc. Nat. Acad. Sci. USA* **70**, 1649-1653 (1973)
- Sabatini, D.D., Bensch, K.G., Barnett, R.J.: Cytochemistry and electron microscopy. The preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation. *J. Cell Biol.* **17**, 19-58 (1963)
- Tokuyasu, K.T., Singer, S.J.: Improved procedures for immunoferritin labeling of ultrathin frozen sections. *J. Cell Biol.* **71**, 894-906 (1976)