Scanning Electron Microscopy

Volume 4
Number 1 *The Science of Biological Specimen Preparation for Microscopy and Microanalysis*

Article 26

1985

Plasma Membrane Antigens Detected by Replica Techniques

H. Hohenberg
Universität Hamburg

W. Bohn
Universität Hamburg

G. Rutter
Universität Hamburg

K. Mannweiler
Universität Hamburg

Science of Biological Specimen Preparation (pp. 235-244)
SEM Inc., AMF O'Hare (Chicago), IL 60666-0507, U.S.A.

PLASMA MEMBRANE ANTIGENS DETECTED BY REPLICA TECHNIQUES

H. Hohenberg*, W. Bohn, G. Rutter, and K. Mannweiler

Heinrich-Pette-Institut für Experimentelle Virologie und Immunologie
an der Universität Hamburg, Martinistrasse 52, 2000 Hamburg 20,
Federal Republic of Germany

Abstract

Methods are introduced for in situ preparation of cell cultures grown on glass coverslips using the replica technique. Special equipment and handling procedures enabled us to prepare large-sized and stable replicas suitable for ultrastructural and immunocytochemical analysis of the different faces of the plasma membrane (PM): the extraplasmic surface (ES), the complementary extraplasmic (EF) and protoplasmic (PF) fracture face, and the protoplasmic surface (PS). Colloidal gold markers in combination with protein A and monospecific/monoclonal antibodies were used to identify virus-specific antigens at the ES of infected cells. Stereo replicas show a coincident location of gold-labeled virus antigens at the ES and structures visible at the EF as well as at the PS. In addition, the association of these antigens with cytoskeletal elements is demonstrated.

KEY WORDS: Cell monolayer, in situ preparation, viral antigens, protein A-gold, surface/fracture label, cytoskeleton, stereo replicas, equipment for replica preparation.

*For reprints and other information please contact:
Heinz Hohenberg at the above address.
Phone No. (040) 477001

Introduction

In recent years the replica technique has become increasingly important for the investigation of biological structures, especially for the three-dimensional demonstration of these structures at high resolution in the transmission electron microscope (TEM).

With this technique fixed and critical point-dried material can be used, and when particular cellular structures have to be examined, the replica technique may be used in connection with quick freezing and deep etching techniques, avoiding the problematic steps of fixation and dehydration (Heuser, 1981). Sometimes it could be necessary to combine the replica method with immunocytochemical procedures, with the purpose of an exact identification of distinct structures. The development of immunogold labeling was an important step in this direction (Faulk and Taylor, 1971; Horisberger and Rosset, 1977).

The electron-dense colloidal gold markers can either be bound to the antigen-antibody complex via protein A (Romano and Romano, 1977) or directly to the antibody (Horisberger, 1981). Such colloidal gold may also be produced in different sizes, thus allowing the size of the gold particles to be adapted to the size of the antigen to be labeled.

We have used replica and labeling techniques to examine virus structures and viral antigens on virus-infected cell cultures. Measles virus, a member of the paramyxo group, was used in the studies performed here. The structural components of this virus are assembled at the apical plasma membrane and are released from the cell by a budding process (Dubois-Dalcq et al., 1984). The virus induces specific structures at the cell surface, which can be clearly identified with the immunogold technique (Mannweiler et al., 1981). Our aim was to examine the mechanism of virus assembly and release at the plasma membrane. However, we encountered several problems in the course of these studies, outlined below:

We intended to perform our studies with cells grown on glass coverslips. For this reason it was important to develop in situ preparation techniques enabling us to demonstrate the plasma membrane surface, the membrane fracture faces after freeze-fracturing, the protoplasmic surface of the apical membrane and the cytoskeletal structures, probably implicated in the release of the virus particles.

The second part of this study deals with handling of replicas up to the stage of mounting on the grid.

With conventional methods (Willison and Rowe, 1980), we encountered problems related to the stability and faithful reproduction of the initial cell material. Thus we have developed techniques with which we are able to prepare replicas in a more controlled, easier and faster manner, eliminating the deformation of replicated structures.

Materials and Methods

Cells and viruses

All experiments presented here were carried out with cells grown as monolayers on glass coverslips. Our studies were performed with HeLa cells chronically or lytically infected with measles virus. Preparations of membranes and cytoskeletons were carried out 20-30 h after infection.

Antibodies

Monoclonal antibodies to measles virus hemagglutinin and rabbit anti-measles antisera were produced in our laboratory (Bohn et al., 1982) and used for surface labeling experiments in combination with protein A-gold (pAg) (Mannweiler et al., 1981).

Protein A-gold system

Colloidal gold with an average particle diameter of less than 10 nm was prepared by ultrasonics as described by Baigent and Müller (1980). 14 nm particles were prepared according to Frens (1973). Gold particles were coated with protein A by use of a microtitration technique (Müller and Baigent, 1980). To make the solution isotonic, 10% sucrose (300 mOsmol) was added to the pAg suspension.

Fixation prior to labeling

Infected cells were fixed with 1% paraformaldehyde and 0.05% glutaraldehyde in 0.2 M phosphate buffer for 20 min at 20°C (Andresen et al., 1981), labeled according to Mannweiler et al. (1981), and postfixed as described below.

Preparation of the different membrane surfaces

Preparation of the extraplasmic surface (ES) (ES: according to freeze-etching nomenclature; Branton et al., 1975) (Fig. 1A). After fixation and labeling, the cultures were rinsed in an exchange apparatus (Baigent et al., 1978) with phosphate buffer, fixed in 1.75% glutaraldehyde for 30 min and in 1% OsO_4 for 60 min, dehydrated in alcohol, passed through Freon 113, critical point-dried with Freon 13 and further processed as described below.

Preparation of freeze-fractured plasma membrane faces, extraplasmic and protoplasmic fracture face (EF and PF). Complementary fracture faces of monolayer cultures were prepared employing the following procedure: Cells were fixed in 1.75% buffered glutaraldehyde for 30 min and incubated in 30% glycerine in phosphate buffer for 1 h. Suitable coverslip areas were selected in the light microscope and excised. Employing a mounted rivet (Fig. 2,1), a suitable coverslip area was frozen to a sandwich in liquid propane or LN_2 slush, freeze-fractured in a double fracture apparatus (Sleytr and Umrath, 1974), and shadowed in the freeze-etch unit Bioetch 2005 (Umrath, 1978). The preparation conditions after freeze fracture of labeled and unlabeled cell cultures differed in the subsequent steps:

Preparation of the EF and PF of unlabeled cells (Fig. 1B). After shadowing, the rivet replica (Fig. 2,1) showing the EF was floated off on water. The shadowed portion of the freeze-fractured cells adhering to the coverslip (coverslip replica see Fig. 2,2) was floated off on hydrofluoric acid. For cleaning away the biological material, both replicas were transferred into a replica washing device (see below).

Preparation of the EF of labeled cells (Fig. 1C). After freeze-fracture and shadowing of the labeled cell monolayer, the rivet replica was thawed and floated directly into the replica washing device. The device contained phosphate buffer that was gradually replaced by 1.75% glutaraldehyde in water and later by water. Biological material was not digested with acid. The labeled outer membrane half remained attached to the Pt/C replica and was directly mounted on a grid. This allowed the simultaneous observation of the surface label and the Pt/C replica of the EF in one single coincident image. The coverslip replica was processed as described in the previous section.

Preparation of the protoplasmic surface (PS) of labeled cells (Fig. 1D). Infected cell monolayers were labeled as described above, stabilized with 0.1% Tannic acid (Rutter et al., 1986), and washed with "internal buffer" (Aggeler et al., 1983). A second coverslip, pre-coated with polylysine or alcian blue (Nermut, 1982), was mounted on top of the labeled coverslip. The two coverslips were gently pressed together and left for 20 min at 4°C. Thereafter, they were separated and the cationized coverslip with the adhering apical membrane was processed for replica preparation as described above.

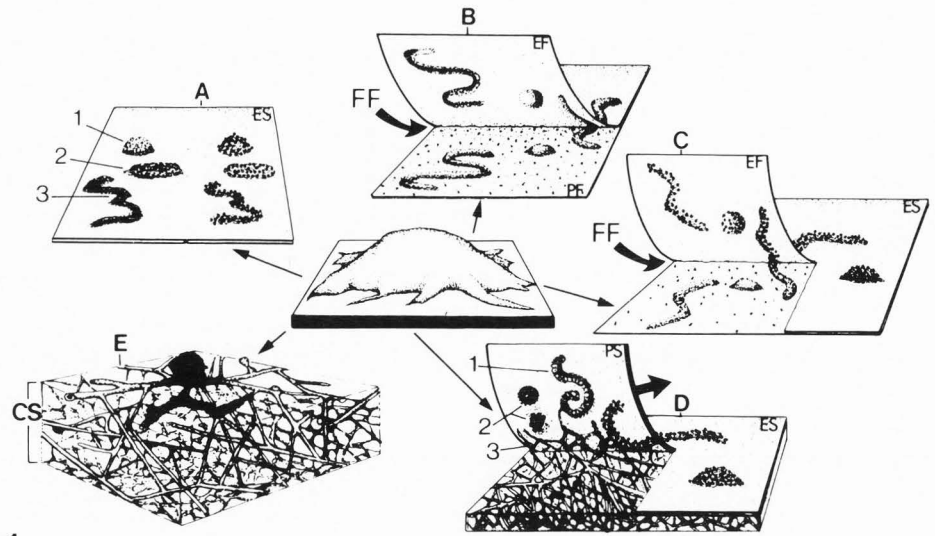
Preparation of the cell cytoskeleton (Fig. 1E)

Virus-infected cells labeled with monoclonal antibodies were lysed with 1% NP-40 in ice-cold buffer containing EGTA and $MgCl_2$ (Bohn et al., 1986), fixed with glutaraldehyde, dehydrated, critical point-dried and shadowed as described in the next section.

Preparation of replicas

Shadowing of all critical point-dried and freeze-fractured samples was done in the freeze-etch unit Bioetch 2005 (Umrath, 1978). The samples, protected by a nitrogen-cooled shroud, were shadowed at -100°C and a pressure $\leq 5 \times 10^{-8}$ mbar, temperature and pressure being constantly monitored. We utilized a resistance evaporator, the evaporation rates of which were regularly measured with a quartz crystal monitor (Veeco). All membranes were shadowed with 2.0-2.5 nm platinum/carbon at a 45° angle and with 15.0-20.0 nm carbon at a 90° angle. Cytoskeleton preparations were shadowed under the same pressure and temperature conditions; however, they were rotated at 30 rpm at angles smaller than 30° and shadowed with platinum/carbon or pure carbon. The carbon backing film was shadowed at angles of 45°-90°.

Detachment of replicas. Shadowed biological material adhering together with the replica to the glass coverslip was separated by floating on 10%



1

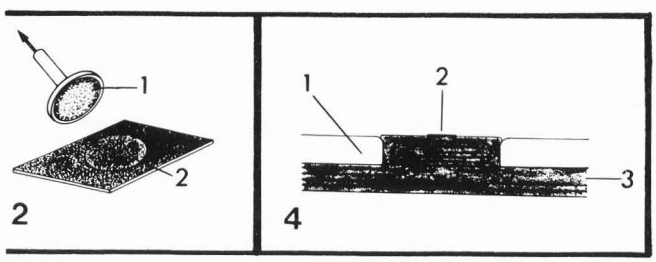


Fig. 1. Schematic drawing of the different preparation steps for platinum/carbon (Pt/C) replication of virus-specific altered plasma membranes (PM) and of cytoskeleton (CS). Central picture: monolayer culture cell grown on glass coverslip. A: Extraplasmic surface (ES), virus-specific structures (left): 1. budding virus, 2. patches, 3. strands. Protein A-gold (pAg)-labeled ES (right). B: Extraplasmic (EF) and protoplasmic (PF) fracture faces after freeze fracture (FF). C: EF and labeled ES after freeze fracture. D: Protoplasmic surface (PS) and labeled ES attached to a second coverslip. 1. Nucleocapsid, 2. clathrin structures, 3. cytoskeleton. E: Labeled virus structures bound to cytoskeleton (CS).

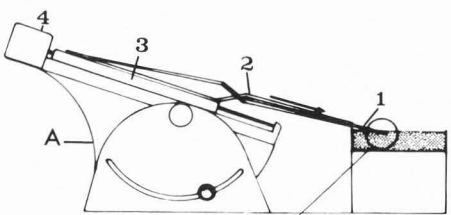
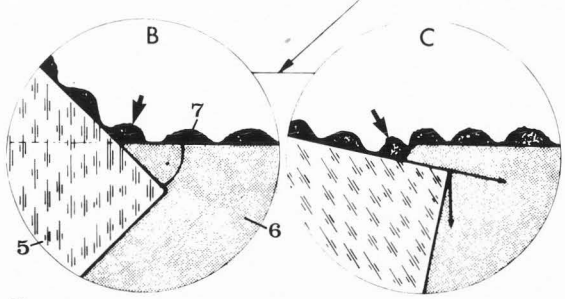


Fig. 2. Freeze fracture of cell monolayer. 1. Rivet replica, 2. coverslip replica.

Fig. 4. Circulating flow in the Teflon compartment of the replica washing device: 1. Teflon, 2. replica, 3. cleaning liquid.



3

Fig. 3. Detachment of replicas. A: Detachment device: A shadowed coverslip (1) clamped between tweezers (2) is immersed into hydrofluoric acid; tweezers are placed on a magnetic holder (3) and moved forward by a motor (4) at low speed. B-C: Faulty detachment. B: Angle at which the coverslip (5) is immersed in the liquid (6) is too steep and produces deformations → at the replica (7). C: Deformations → caused by too rapid immersion → or by vertical movement ↓.

hydrofluoric acid. If specimens were highly fragile or required more time for separation, we used a device developed in our laboratory as described in Fig. 3A. Using this device, the shadowed material was floated on to the surface of hydrofluoric acid at an angle smaller than 30° in order to avoid deformations (Fig. 3B). Immersion was done as rapidly as it took the replica to become detached, thus preventing submersing or breaking it, as shown in Fig. 3C. In the case of large shadowed areas, the film was divided into 3.5 x 3.5 mm squares prior to floating.

Cleaning of replicas. For removing the biological matrix, the replicas were transferred from the floating vessel by means of a platinum loop into the compartments of an automatic washing device (Balzers, Liechtenstein) developed in our laboratory (Hohenberg and Mannweiler, 1980). The washing device permits cleaning of several replicas under identical conditions while they are floating on the surface of the cleaning liquid (30% chromic acid). The special construction of the compartments in a Teflon disc (see Fig. 4) ensures that the biological material is gently washed out from below in a gradient of chromic acid without subjecting the replica to any mechanical stress. After cleaning, the replicas were mounted on a grid without any need for a supporting film.

Cleaning of the cytoskeletons and the replicas of protoplasmic surface (PS). Cytoskeletons and replicas of the PS were floated on hydrofluoric acid as described above without removing the biological material and rinsed in the washing device with water from below to remove the acid. Subsequently, they were mounted on grids without any supporting film and examined in the TEM (Philips EM 400 T).

Mounting of replicas. The replicas were mounted on the shiny flat surface of hexagonal grids. They were dried "headover" by turning the grid upside down and drawing the water cautiously from the rough side of the grid.

The different steps of replica preparation described above were controlled by the following procedure (Hohenberg et al., 1981): Coverslip cultures were prepared as described above and shadowed with Pt/C. Pieces of the shadowed coverslip were fastened to a SEM specimen holder with conducting silver. Selected areas were examined and photographed in the SEM (Cambridge S 4-10) at an angle of 45°. Thereafter, the replicas were detached as described, cleaned, mounted on grids and re-examined at the same angle in the SEM, but this time without biological substrate. By comparing the pictures of the first and the second SEM examination, we were able to determine the degree of reproduction fidelity.

Results and Discussion

Labeling of virus-specific antigens on the ES

Measles virus-infected cells treated with antibodies to hemagglutinin and with protein A-gold show a distinct labeling of virus-specific structures at the cell surface. Because of the small size of the markers, the virus-specific alterations of the plasma membrane (Figs. 5a,b) are morphologically still visualized after labeling (Figs. 6 and 7). Experiments with even smaller gold particles between 4 and 7 nm have demonstrated that these are not suited for the replica technique. Because of the high contrast of the platinum/carbon film, they are no longer clearly identifiable.

Controls were done with normal cells treated as infected ones and with infected cells incubated with normal rabbit serum instead of specific antibodies. No labeling could be demonstrated.

Virus-induced alterations of EF/PF morphology

With the in situ freeze-fracture method we are able to produce large areas of complementary fracture faces of the plasma membrane. Pictures of the EF and PF show that on both membrane faces there are altered areas with a patched (Fig. 8) or band-like (Fig. 9) arrangement. The patches at the PF are devoid of those intramembranous particles which are found in the neighboring areas (Fig. 8, 1). The particles within these structures are extremely small and appeared to have no regular or crystalline arrangement. Freeze-fractured virus (Fig. 8,2) has the same fine structure as seen in the patches. These altered areas at the PF are convex and could possibly be determined in their shape by nucleocapsids adhering to the corresponding PS. The band-like structures at the EF show a higher density of intramembranous particles than the neighboring areas; they are concave and similar in shape and size to the virus-specific structures, which were labeled at the ES of the plasma membrane. Direct demonstration of the coincidence of altered intramembranous morphology with virus antigens at the ES or nucleocapsids at the PS was not possible with the method described above.

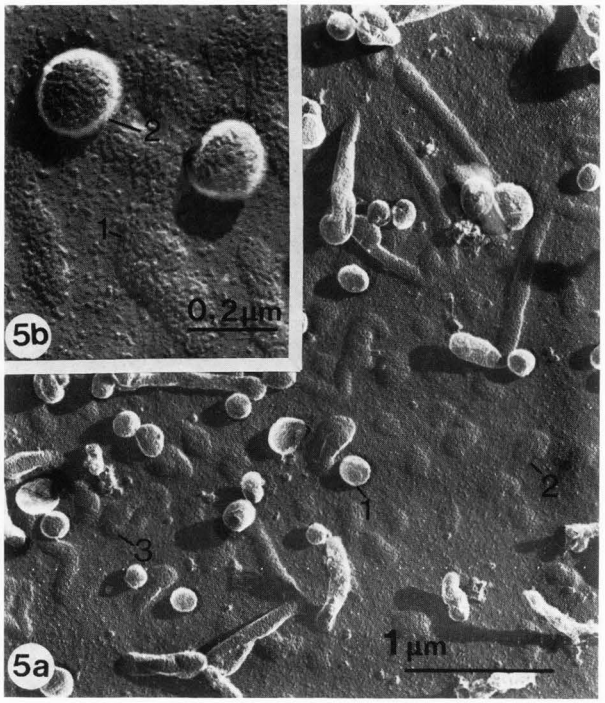
Morphology of the EF and ES labeling at the same replica

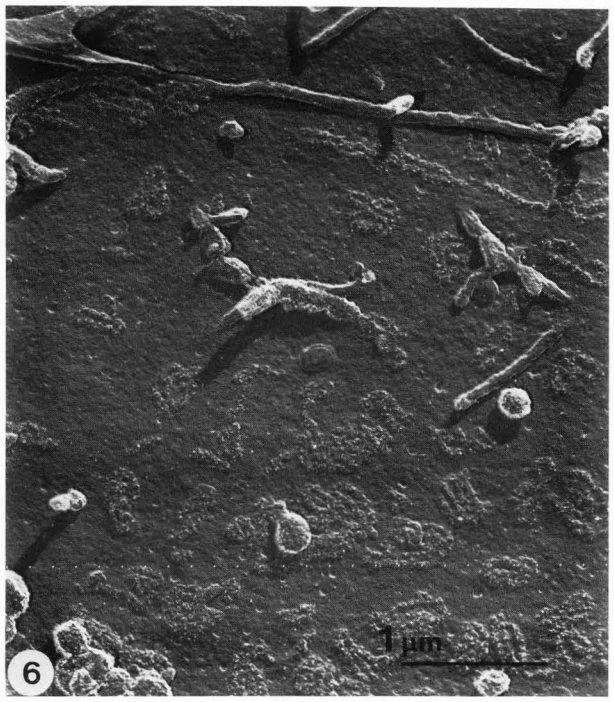
A direct correlation between labeled antigens at the cell surface and the morphology of the EF was made possible due to a method developed by Pinto da Silva and Kan (1984). We have modified this method for virus-infected cell monolayers with the following results: The concave strands and patches on the EF of unlabeled cells (Figs. 8 and 9) are also present in labeled membrane preparations, but now the labeled ES antigens could be observed on the Pt/C replica of the EF. A direct correlation between distribution of ES antigens and intramembranous particle density is possible. Stereo pictures (Fig. 10) demonstrate that the distribution of the gold marker proved to be strongly correlated to these concave structures.

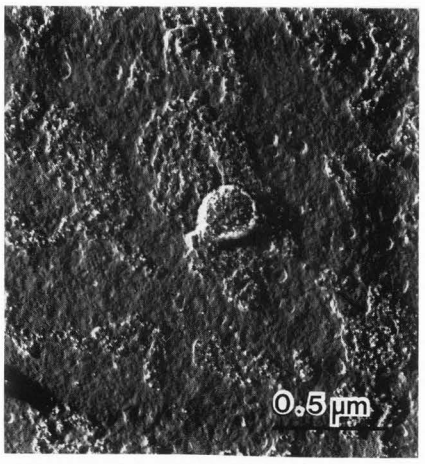
In some regions (Fig. 11), in addition to the contrast of the gold marker and the Pt/C film, there is also a contrast of cell material or budding virus present on the cell surface. Such regions are so electron-dense that the EF fine structure is no longer visible. However, this method permitted us to investigate the distribution patterns of cell surface antigens and their relationship to membrane architecture.

Morphology of the PS and ES labeling at the same replica

The described method is very efficient in producing numerous apical plasma membranes favorably arranged for the examination of their protoplasmic surface (Fig. 12). Mainly nucleocapsids are seen attached at the PS as well as cellular structures like clathrin, coated regions and cytoskeletal elements (Fig. 13). Nucleocapsids were clearly identified by labeling with monoclonal antibodies against nucleocapsid associated proteins (Rutter et al., 1984). Figure 13 shows an unequivocal coherence between the virus antigens labeled at the ES and the nucleocapsids at the PS; this permits the study of virus assembly at the cell membrane and budding from the cell membrane.







Figs. 5-13. Pt/C surface replicas of measles virus-specific structures on HeLa cells in the TEM.

Figs. 5-7: Replicas of the ES (reversed prints). Fig. 5a: Accumulation of virus-specific structures: (1) budding virus, (2) patches, (3) strands. Fig. 5b: Higher magnification of (1) specific granular structures, (2) budding formation of virus particle. Fig. 6: Immunogold labeling of viral structures; labeling with antibodies to hemagglutinin and with protein A-gold. Fig. 7: Stereo pictures of labeled ES; enlarged detail of Fig. 6.

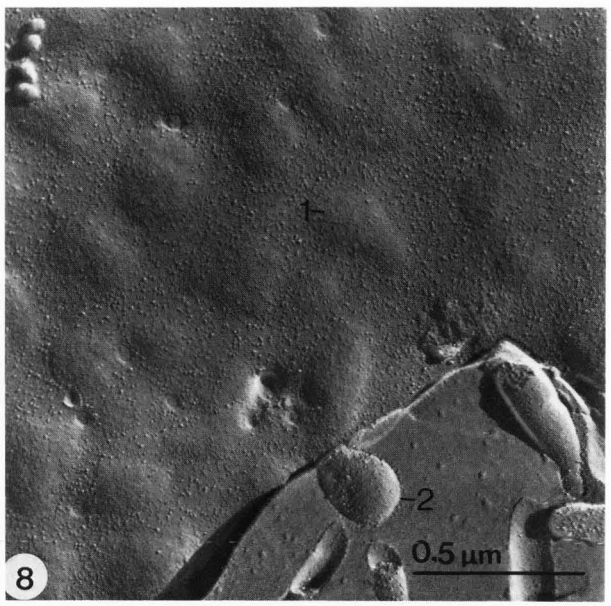
Association of virus structures with the cytoskeleton

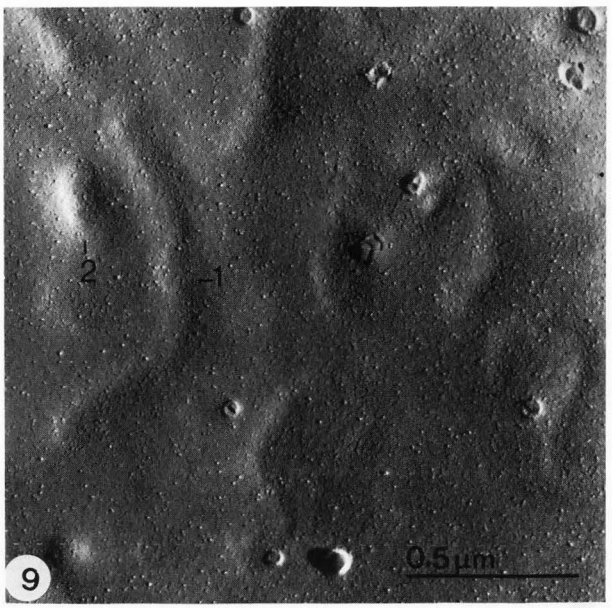
Recent studies on measles virus morphogenesis have pointed to an involvement of cytoskeletal structures in the formation of budding particles at the plasma membrane (Bohn et al., 1983, 1986).

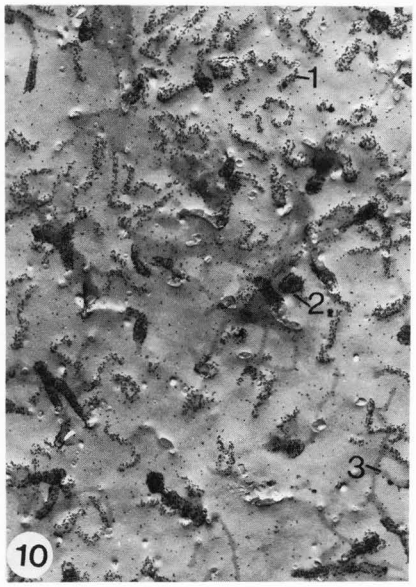
Cytoskeletons were prepared from infected cells by extraction with a nonionic detergent. Stereo pictures of shadowed cytoskeletons clearly reveal the presence of labeled budding particles (Fig. 14) bound to cytoskeletal filaments. The virus particles have maintained their normal morphology.

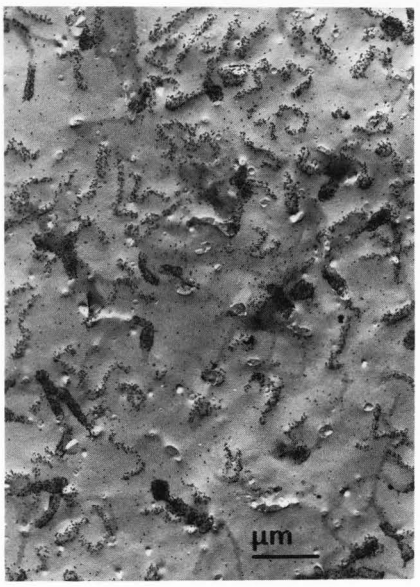
With this method the association of budding particles with the submembranous cytoskeletal network could be visualized.

The morphological studies point to a specific interaction of viral structures with the cytoskeleton in the course of virus release.





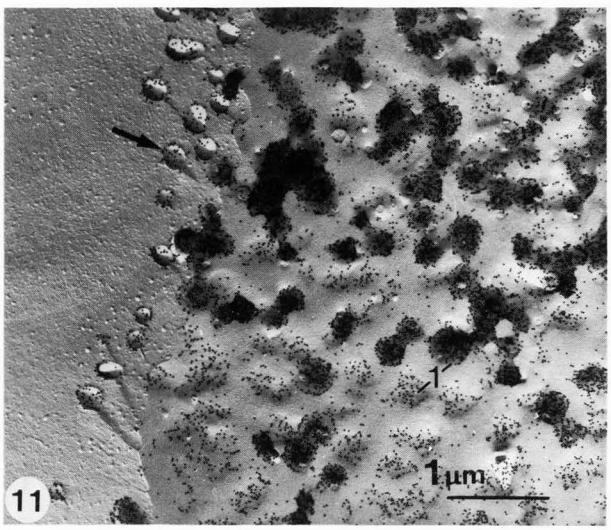


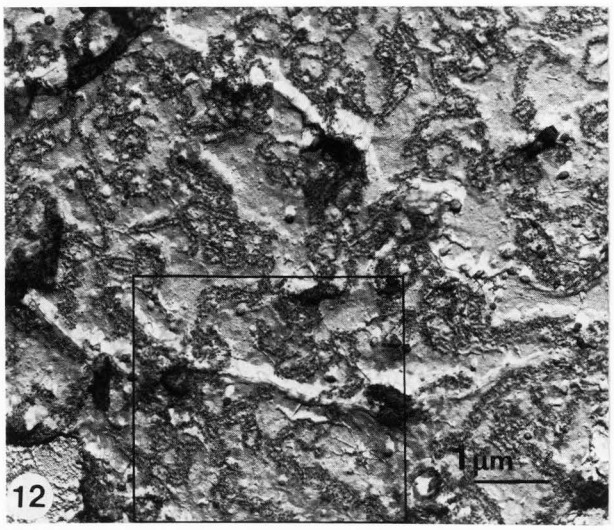


Figs. 8-11: Replicas of freeze-fractured plasma membranes. Fig. 8: Specific alterations of the PF (reversed print). (1) Convex patches devoid of particles found in the neighboring membrane areas, (2) freeze-fractured budding virus in the extracellular space. Fig. 9: Specific alterations of the EF (reversed print). (1) Concave strands or (2) patches with higher density of small particles. Fig. 10: Stereo pictures of the plasma membrane labeled on the ES and freeze-fractured; replica of the EF. (1) Concave strands or (2) budding virus with additional material contrast, (3) material contrast of surface ruffles and microvilli. Fig. 11: Replica of the EF and labeled ES showing different labeling densities in areas of low and high material contrast (1); labeled and freeze-fractured budding virus in the extracellular space (➡).

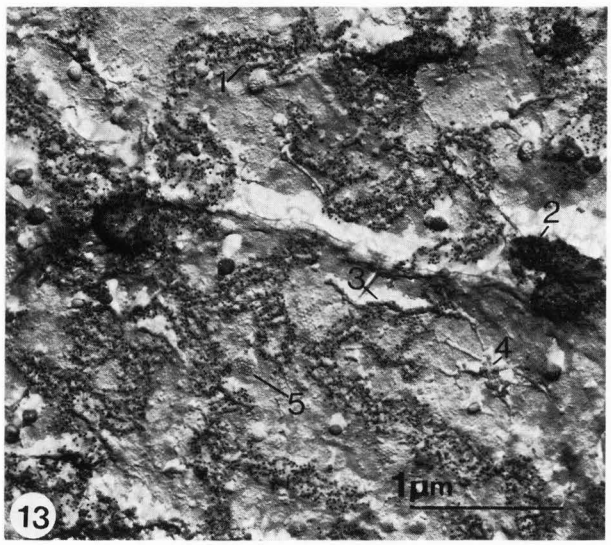
Preparation of replicas

As already shown (Steere, 1982), high resolution shadow films made of a mixture of Pt and C can be very reliably deposited from resistance evaporators. However, it is very important that there is a clean vacuum in the vicinity of the specimen (Sleytr and Messner, 1978). A further factor influencing the quality of the Pt/C films is the temperature of the specimen itself (Peters, 1984). For this reason we shadowed all samples at the same temperature at which the freeze-fracturing was performed. The Pt/C films produced under these conditions are resistant to the acid concentrations used to remove the biological material. The protein A-gold complexes were so strongly bound to the shadowed matrix that they stay anchored in the Pt/C film after the replica washing





See facing page for the caption of Fig. 11.



Figs. 12-13: Pictures of isolated membranes labeled on the ES. Fig. 12: Replica of the PS. Overview showing nucleocapsids at the PS and extraplasmic surface labeling in one coincident image. Fig. 13: Higher magnification of Fig. 12. (1) Nucleocapsids, (2) budding virus with dense pAg-labeling and additional material contrast, (3) nucleocapsid without labeling on the ES, (4) adhering cytoskeletal structures, (5) clathrin structures.

procedure. For some cytoskeleton preparations we used also a Pt/C rotation evaporation at an angle of ≦ 30° for accurate measurement of cytoskeletal structures. Other preparations of labeled cytoskeletons were only shadowed with carbon for a better identification of gold labeling.

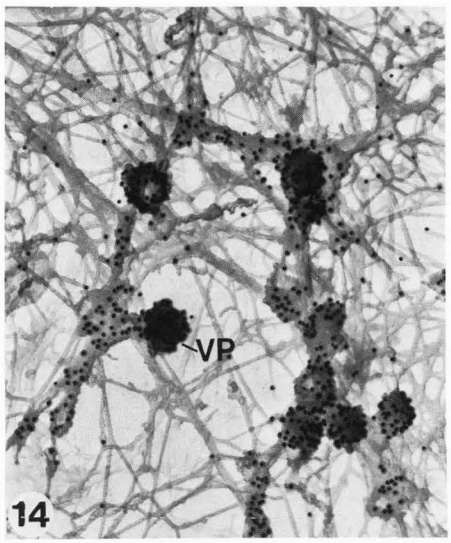
Comparative studies of shadowed cytoskeletons and cells in the SEM prior to and after replica preparation have revealed severe deformations during detachment of the sample from the substrate. The detachment device presented here enabled a more controlled and easier detachment of replica and biological material than could be achieved manually. This applied in particular to specimens difficult to detach from the substrate, where the process of detachment took more time. Applying the described detachment conditions, we obtained, for instance, cytoskeleton replicas which after detachment were fully preserved and could be mounted on a grid without backing film to be examined in the TEM (Fig. 15).

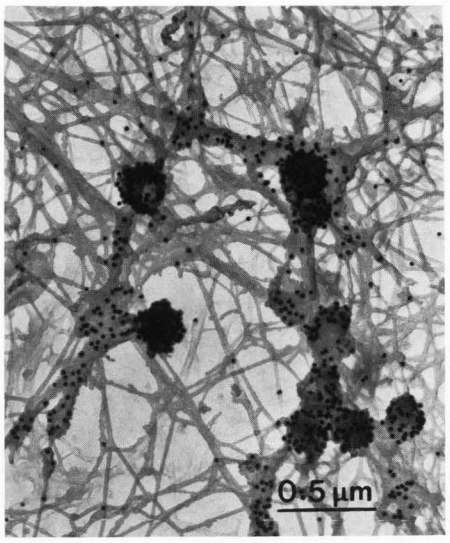
If the replicas were cleaned according to standard procedures (Willison and Rowe, 1980), i.e., by transfer of individual replicas from one washing vessel to another by means of a platinum loop, fragile ones inevitably broke apart. This complicated the preparation of complementary replicas or preparations of selected cell areas. Furthermore, with conventional cleaning procedures only such replicas survived which were stable enough because they were very flat, having no highly irregular surfaces.

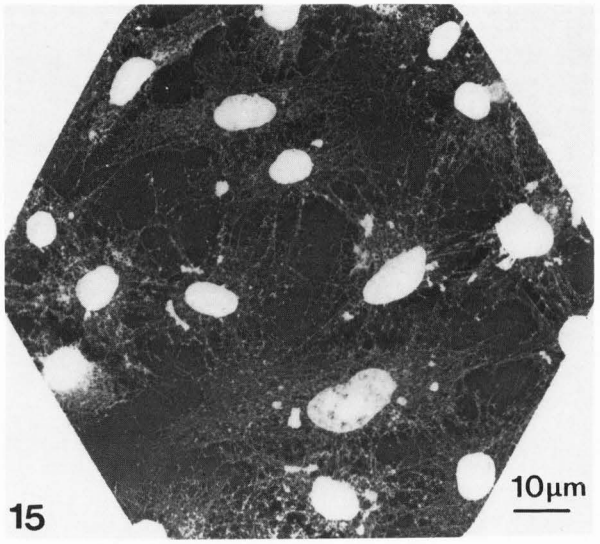
These difficulties are overcome by using the replica washing device. In this device the replicas are subjected to fully automatic cleaning without the occurrence of turbulence. Transfer of the replica is no longer necessary and thus, even highly fragile replicas can be recovered in a well preserved condition from the device after removal of the biological material.

It has been our experience that replicas which are mounted on the flat shiny surface of a hexagonal grid will show the least tears due to tension in the course of drying. Other grid forms, for instance square types, produced diagonal tears in sensitive material, which rendered investigations in the TEM high-resolution range very difficult due to the film drift. The rough grid surface caused the replica to cling to it and to tear when thermic stresses arose.

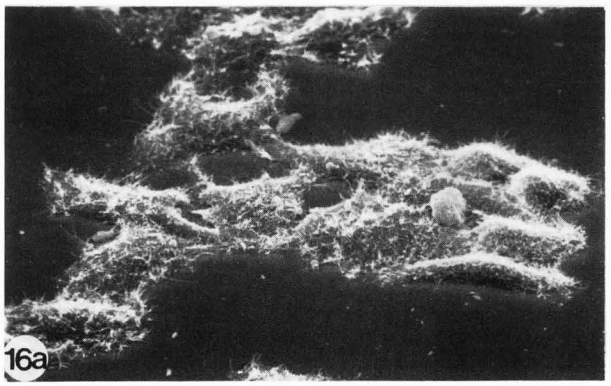
"Headover" drying prevents replicas with irregular topography from collapsing, because they are stabilized by the weight of the water until the end of the drying process. Backing films underneath the replica are not required, avoiding reduction of contrast and preventing deformations







Figs. 14-15. Rotation shadowed cytoskeletons. Fig. 14: Stereo pictures of carbon shadowed cytoskeleton with labeled budding virus particles (VP). Fig. 15: Pt/C shadowed cytoskeletons in TEM without any supporting film.



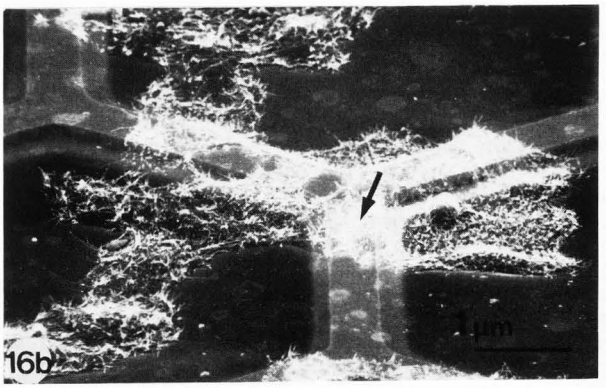


Fig. 16. Pt/C shadowed HeLa cells in the SEM. a. Before, b. after the replication procedure, (➞) deformation of replicas at the grid stages.

caused by capillary effects between stabilization film and replica during the drying procedure. Similar deformation effects were seen in the SEM when replicas dried on grid bars (Fig. 16b). Generally, to determine the degree of reproduction fidelity, it was very helpful to compare the cell surface topography in the SEM prior to (Fig. 16a) and after (Fig. 16b) replica preparation procedures.

Conclusion

In order to obtain high-quality replicas of immunolabeled cell surfaces, one has to be aware of the problems in immunolabeling (such as inactivation of antigens by fixation, specificity of the antigen-antibody reaction, preparation and coating of colloidal gold). These questions were extensively reviewed by others (Polak and Varndell, 1984) and therefore are not discussed here in detail. Here we mainly referred to the critical steps in preparing and handling replicas of labeled membranes.

With the methods described here we were able to obtain large stereo replicas with high stability from cells grown on glass coverslips, the substrate normally used in cell culture techniques. The data show that a combination of immunogold and replica technique is suitable for the localization of plasma membrane antigens at high resolution in TEM. Furthermore, a correlation could be made between the localization of surface antigens and structures seen on freeze-fractured membranes and on the PS of isolated membranes. In addition, the association of these antigens with cytoskeletal elements could be demonstrated. The data exclusively refer to labeling of viral antigens. However, the methods may also be suitable for studies on surface antigens or cellular receptors in future studies.

Acknowledgements

This work was supported in part by the Gemeinnützige Hertie-Stiftung. The Heinrich-Pette-Institut is financially supported by Freie und Hansestadt Hamburg and Bundesministerium für Jugend, Familie und Gesundheit.

References

Aggeler J, Takemura R, Werb Z. (1983). High-resolution three-dimensional views of membrane-associated clathrin and cytoskeleton in critical-point-dried macrophages. J. Cell. Biol. 97, 1452-1458.

Andresen I, Mannweiler K, Bohn W, Hohenberg H, Rutter G, Ueckermann C. (1981). Influence of preparation conditions on antigen labeling by gold markers. Investigations with replicas and ultrathin sections. Beitr. elektronenmikroskop. Direktabb. Oberfl. 14, 437-444.

Baigent CL, Mannweiler K, Andresen I, Rutter G, Neumayer U. (1978). Improved and simplified "exchange procedure" for the preparation of infectious coverslip cultures for comparative SEM and TEM studies. Beitr. elektronenmikroskop. Direktabb. Oberfl. 11, 251-262.

Baigent CL, Müller G. (1980). A colloidal gold prepared with ultrasonics. Experientia (Basel) 36, 472.

Bohn W, Rutter G, Mannweiler K. (1982). Production of monoclonal antibodies to measles virus proteins by immunization with heated and detergent-treated antigens. Virology 116, 368-371.

Bohn W, Rutter G, Hohenberg H, Mannweiler K. (1983). Inhibition of measles virus budding by phenothiazines. Virology 130, 44-55.

Bohn W, Rutter G, Hohenberg H, Mannweiler K, Nobis P. (1986). Involvement of actin filaments in budding of measles virus. Studies on cytoskeletons of infected cells. Virology 149, 91-106.

Branton D, Bullivant S, Gilula NB, Karnovsky MJ, Moor H, Muhletaler K, Northcote DH, Packer L, Satir B, Satir P, Speth V, Staehelin LA, Steere RL, Weinstein RS. (1975). Freeze etching nomenclature. Science 190, 54-56.

Dubois-Dalcq M, Holmes KV, Rentier B, Kingsbury DW (eds). (1984). Assembly of Enveloped RNA Viruses. Springer-Verlag, Wien.

Faulk WP, Taylor GM. (1971). An immunocolloid method for the electron microscope. Immunochemistry 8, 1081-1083.

Frens G. (1973). Controlled nucleation for the regulation of the particle size of monodisperse gold suspensions. Nature Phys. Sci. 241, 20-22.

Heuser J. (1981). Quick-freeze, deep-etch preparation of samples for 3-D electron microscopy. Trends Biochem. Sci. 6, 64-68.

Hohenberg H, Mannweiler K. (1980). Semi-automatic washing device for simultaneous cleaning of surface replicas under identical conditions. Mikroskopie (Wien) 36, 145-154.

Hohenberg H, Mannweiler K, Neumayer U, Ueckermann C. (1981). A preparation method to demonstrate identical biological objects in the SEM and subsequently, by replica procedure, in the TEM. Studies on C.P.-dried coverslip cell cultures. Beitr. elektronenmikroskop. Direktabb. Oberfl. 14, 445-454.

Horisberger M, Rosset J. (1977). Colloidal gold, a useful marker for transmission and scanning electron microscopy. J. Histochem. 25, 295-305.

Horisberger M. (1981). Colloidal gold: a cytochemical marker for light and fluorescent microscopy and for transmission and scanning electron microscopy. Scanning Electron Microsc. 1981; II: 9-31.

Mannweiler K, Hohenberg H, Bohn W, Rutter G. (1981). Protein-A gold particles as markers in replica immunocytochemistry: high resolution electron microscope investigations of plasma membrane surfaces. J. Microsc. 126, 145-149.

Müller G, Baigent CL. (1980). Antigen controlled immuno-diagnosis - 'Acid test'. J. Immunol. Meth. 37, 185.

Nermut MV. (1982). The 'cell monolayer technique' in membrane research: a review. Eur. J. Cell. Biol. 28, 160-172.

Peters K-R. (1984). Continuous ultrathin metal films, in: Science of Biological Specimen Preparation, J.P. Revel, T. Barnard, G.H. Haggis (eds), SEM Inc. AMF O'Hare, Chicago, Illinois, pp. 221-231.

Pinto da Silva P, Kan FWK. (1984). Label-fracture: a method for high resolution labeling of cell surfaces. J. Cell Biol. 99, 1156-1161.

Polak JM, Varndell IM (eds). (1984). Immunolabeling for Electron Microscopy. Elsevier Science Publishers B.V., Amsterdam.

Romano EL, Romano M. (1977). Staphylococcal protein A bound to colloidal gold: a useful reagent to label antigen-antibody sites in electron microscopy. Immunochemistry 14, 711-715.

Rutter G, Bohn W, Hohenberg H, Mannweiler K. (1984). Preparation of apical plasma membranes from cells grown on coverslips. Studies on the cytoplasmic face of virus infected cells, in: Proc. 8th Eur. Congr. Electron Microscopy, Budapest, A. Csanády, P. Röhlich, D. Szabó (eds), pp. 1850-1860.

Rutter G, Bohn W, Hohenberg H, Mannweiler K. (1986). Preparation of apical plasma membranes from cells grown on coverslips. Electron microscopic investigations of the protoplasmic surface. Eur. J. Cell Biol. 39, 443-448.

Sleytr UB, Umrath W. (1974). A simple fracturing device for obtaining complementary replicas of freeze-fractured and freeze-etched suspensions and tissue fragments. J. Microsc. 101, 177-186.

Sleytr UB, Messner P. (1978). Freeze fracturing in normal vacuum reveals ringlike yeast plasmalemma structures. J. Cell Biol. 79, 276.

Steere RL. (1982). Reliable use of resistance evaporation of Pt and C for high resolution freeze-fracturing and a crystal surface image complementary to the E-face of yeast plasma membranes. J. Microsc. 128, 157-166.

Umrath W. (1978). Automatische Gefrierätzung. Mikroskopie (Wien) 34, 6-10.

Willison JHM, Rowe AJ. (1980). Replica, shadowing and freeze-etching techniques, in: Practical Methods in Electron Microscopy, A.M. Glauert (ed), Elsevier/North-Holland Biomedical Press, pp. 155-158.