Histol Histopathol (2000)15: 515-522

http://www.ehu.es/histol-histopathol

Histology and Histopathology
Cellular and Molecular Biology

Invited Review

Freeze-fracture cytochemistry: a new fracture-labeling method for topological analysis of biomembrane molecules

T. Takizawa[1] and J.M. Robinson[2]

[1]Department of Anatomy, Jichi Medical School, Tochigi, Japan and

[2]Department of Physiology and Cell Biology, Ohio State University, Columbus, OH, USA

Summary. Freeze-fracture cytochemistry allows visualization of cellular and molecular characteristics of biomembranes *in situ*. In this review, we discuss freeze-fracture cytochemistry with special reference to a new cytochemical labeling of replicas, the detergent-digestion fracture-labeling technique. In this procedure, unfixed cells are rapidly-frozen, freeze-fractured, and physically stabilized by evaporated platinum/carbon. The frozen cells are then removed from the freeze-fracture apparatus to thaw and are subsequently treated with detergents. After detergent-digestion, replicas are labeled with cytochemical markers. We demonstrate that the technique is a versatile tool for direct analysis of the macromolecular architecture of biomembranes and allows identification of particular intracellular membrane organelles. In addition, we demonstrate the application of ultrasmall gold to freeze-fracture immunocytochemistry. Freeze-fracture cytochemistry is a valuable technique for investigating topology and dynamics of membrane molecules.

Key words: Freeze-fracture electron microscopy, Immunocytochemistry, Enzyme cytochemistry, Ultra-small immunogold, Neutrophils

Introduction

The combination of freeze-fracture electron microscopy and cytochemistry ("freeze-fracture cytochemistry") provides unique information concerning morphological analysis of biomembranes that can not be readily obtained using conventional cytochemistry alone. Freeze-fracture electron microscopy provides high resolution morphological information; freeze-fracture cytochemistry provides additional information on *in situ* expression of biomembrane components. Several types of freeze-fracture cytochemistry have been described based on the cytochemical method used. These include: (1) freeze-fracture autoradiography (Fisher and Branton, 1976; Rix et al., 1976; Carpentier et al., 1985), (2) freeze-fracture cholesterol cytochemistry (Verkleij et al., 1973; Elias et al., 1979), (3) freeze-fracture carbohydrate cytochemistry (Pinto da Silva et al., 1981c), (4) freeze-fracture immunocytochemistry (Pinto da Silva et al., 1981a), and (5) freeze-fracture enzyme cytochemistry (Takizawa and Saito, 1996, 1997; Takizawa et al., 1997). Hence, freeze-fracture cytochemistry allows characterization of many types of membrane molecules.

Application of this method has advanced our understanding of various aspects of cellular biology. Immunocytochemistry has been the most widely applied technique in the area of freeze-fracture cytochemistry. Recent technical advances in this field have focused on immunocytochemical labeling after detergent digestion of half-membrane leaflets stabilized by platinum/carbon (Pt/C) evaporation (Fujimoto, 1995, 1997; Takizawa et al., 1998). In this review, we describe the fundamental principles of the new freeze-fracture replica labeling technique developed in our own laboratories and its use for detection of specific antigen molecules as well as for detection of enzyme activity. We also review our work on the application of an ultrasmall immunoprobe to freeze-fracture immunocytochemistry.

Fracture-label and label-fracture

From the cytochemical viewpoint, the origin of the detergent-digestion fracture-labeling technique dates back to the early 1980s, although today's freeze-fracture electron microscopy is based on the pioneering work of Hall (1950). The "fracture-label" technique was first introduced for detection of carbohydrates in biological membranes (Pinto da Silva et al., 1981a-c). The method was named "fracture-label" based on the sequence of the procedure; chemically fixed samples were freeze-fractured first and then labeled with various cyto-

Offprint requests to: Toshihiro Takizawa, MD, PhD, Department of Anatomy, Jichi Medical School, 3311 Yakushiji, Minamikawachi-machi, Tochigi 329-0498, Japan. Fax: +81-285-44-7067. e-mail: ttakizawa@jichi.ac.jp

chemical markers (e.g., cationized ferritin, wheat germ agglutinin-conjugated colloidal gold particles). The resultant views of the two-dimensional distribution of particular membrane molecules allow us to determine regional biochemical properties of cellular membranes. However, the method has certain technical limitations: thawing prior to cytochemical reactions causes significant modifications of split membrane halves since those membranes had not been Pt/C stabilization (for reviews see Pinto da Silva, 1987; Severs, 1991; Torrisi and Mancini, 1996). That method was followed by the development of the "label-fracture" technique, in which cell surfaces are cytochemically labeled and then followed by freeze-fracture replication (Pinto da Silva and Kan, 1984). In the label-fracture technique, the routine replica-cleaning procedure using sodium hypochlorite is omitted since cytochemical markers as well as unfractured cell components are readily dissociated from replica membranes by strong cleaning reagents (Pinto da Silva, 1987; Severs, 1991; Torrisi and Mancini, 1996). Whereas its application has been limited primarily to cell surfaces [i.e., the exoplasmic halves (E-faces) of cell membranes], the technique implied the utility of the split membrane halves followed by Pt/C evaporation for freeze-fracture cytochemistry. However, to allow extensive views of intracellular membrane systems [e.g., the protoplasmic half (P-face) of cell membranes, nuclear membranes] as well as cell surfaces and label particular membrane molecules with cytochemical markers, it is necessary to overcome the following obstacles: (1) freeze-fractured membrane halves should be physically stabilized prior to the thawing of samples; (2) only unfractured cell components should be extracted from replicated samples; (3) cytochemical probes should penetrate into sites containing targeted molecules within cells.

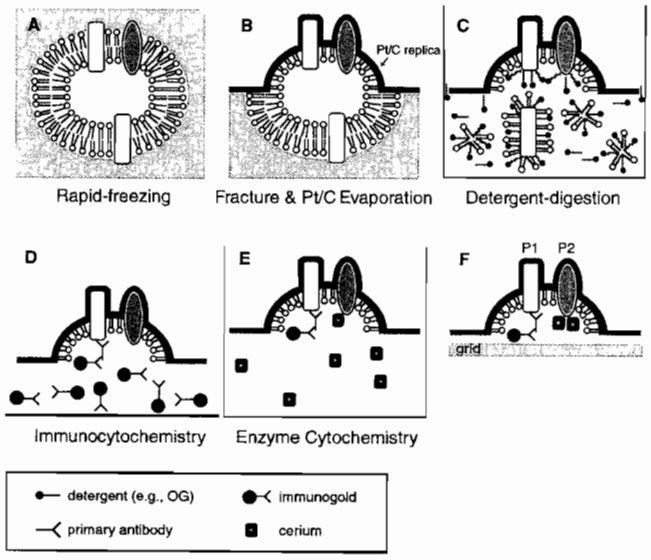

Fig. 1. Diagrammatic representation of the procedure of "detergent-digestion fracture-labeling technique". Unfixed sample is rapidly frozen **(A)**, freeze-fractured, and then physically stabilized with evaporated Pt/C **(B)**. Sample is digested with an appropriate detergent [e.g., octyl-glucoside (OG)] to solubilize unfractured cell components **(C)**. This detergent is gentle enough to preserve Pt/C-stabilized membrane halves yet sufficient to extract unfractured membranes and cytoplasm. After detergent-digestion, the Pt/C replica, along with attached biomembrane half, is labeled by immunocytochemistry **(D)** and subsequent enzyme cytochemistry **(E)** and mounted on a formvar-coated EM grid **(F)**. The Pt/C-stabilized membrane half is labeled with two different cytochemical markers; in this case one protein (P1) is detected with an immunogold particle while the second (P2) is demonstrated with cerium reaction product.

Detergent-digestion fracture-labeling technique

"Detergent-digestion fracture-labeling technique" was initially described by Fujimoto and Pinto da Silva (1992). In this method, unfixed cells and tissues are first frozen quickly, freeze-fractured, and physically stabilized with evaporated Pt/C. Samples are then digested with appropriate detergents to solubilize unfractured cell components. Finally, after detergent-digestion, the Pt/C replicas, along with attached biomembrane halves, are labeled with cytochemical probes, followed by electron microscopic examination (Fig. 1). This technique is well suited for freeze-fracture immunocytochemistry and to overcome the obstacles mentioned above. In this method, the use of unfixed cells is crucial because cells chemically fixed with aldehydes (e.g., glutaraldehyde, paraformaldehyde) are not readily digested with detergents. On the other hand, unfixed cells are extracted by detergent treatment [e.g., Triton X-100 and sodium dodecyl sulfate (SDS)] (Fujimoto and Pinto da Silva, 1992; Fujimoto, 1995; Fujimoto et al., 1996; Takizawa and Saito, 1997). The latter detergent is the well-known dissociating agent used in polyacrylamide gel electrophoresis. Using quantitative analysis of artificial multilamellar vesicles containing phosphatidylcholine (PC), Fujimoto and associates estimated that at least 70% of PC in the freeze-fractured membrane halves that were stabilized by Pt/C evaporation remained after SDS-digestion (Fujimoto et al., 1996). These findings provide evidence that the hydrophobic fracture face of split membrane halves are "fixed" with the Pt/C replicas, and thus most, if not all, constituents of the membrane halves remain attached to the replicas even after detergent-digestion.

Introduction of octyl-glucoside as a detergent for detergent-digestion fracture-labeling technique

To further understand the macromolecular organization of biomembranes, multi-labeling techniques are of particular importance. Immunocytochemical double labeling using immunogold particles of various sizes has been reported (e.g., Slot and Geuze, 1981; Fujimoto, 1995). The combination of immunocytochemistry and enzyme cytochemistry would be desirable in certain experimental situations. Although SDS is an efficient detergent for freeze-fracture immunocyto-

chemistry, SDS-digestion results in a significant loss of biological activity, such as enzyme activity, from Pt/C-stabilized membrane halves. SDS, a strong ionic detergent, probably denatures native enzyme molecule structures on the membrane halves, thereby making them inactive and unusable for enzyme cytochemical studies (Takizawa et al., 1998). Therefore, instead of SDS, a more suitable compound should be used in this method for replica digestion (Takizawa and Saito, 1997; Takizawa et al., 1998).

Detergent-digestion fracture-labeling technique has been extended by the use of octyl-glucoside (OG) (Takizawa et al., 1998). OG-digestion, in addition to being an efficient detergent, preserves not only immunocytochemical antigenicity but also enzyme activity on Pt/C-stabilized membrane halves. Thus, OG-digestion can reveal the relationship between molecules in biological membranes by double-labeling with two different cytochemical markers (i.e. immunogold probes for immunocytochemistry and cerium for enzyme cytochemistry). OG, a nonionic detergent, was initially reported as an effective biochemical agent for solubilizing membrane proteins (Baron and Thompson, 1975).

The method is summarized in Figure 1. Pt/C-replicated frozen-samples are treated with OG. In OG-digestion, the unfractured cellular components are solubilized while the fractured membrane stabilized by Pt/C remains. After OG-digestion, the replicas can be labeled by immunocytochemistry or enzyme cytochemistry, or double-labeled with both procedures.

The critical micelle concentration (CMC) of the detergent is a key consideration in successful fracture-labeling. CMC is defined as the concentration at which detergents begin to associate to form micelles in solution; the CMC of OG is 20-25 mM, while that of SDS is 8.2 mM (Shinoda et al., 1961; Helenius and Simons, 1975; Gould et al., 1981). The detergent concentration has a greater

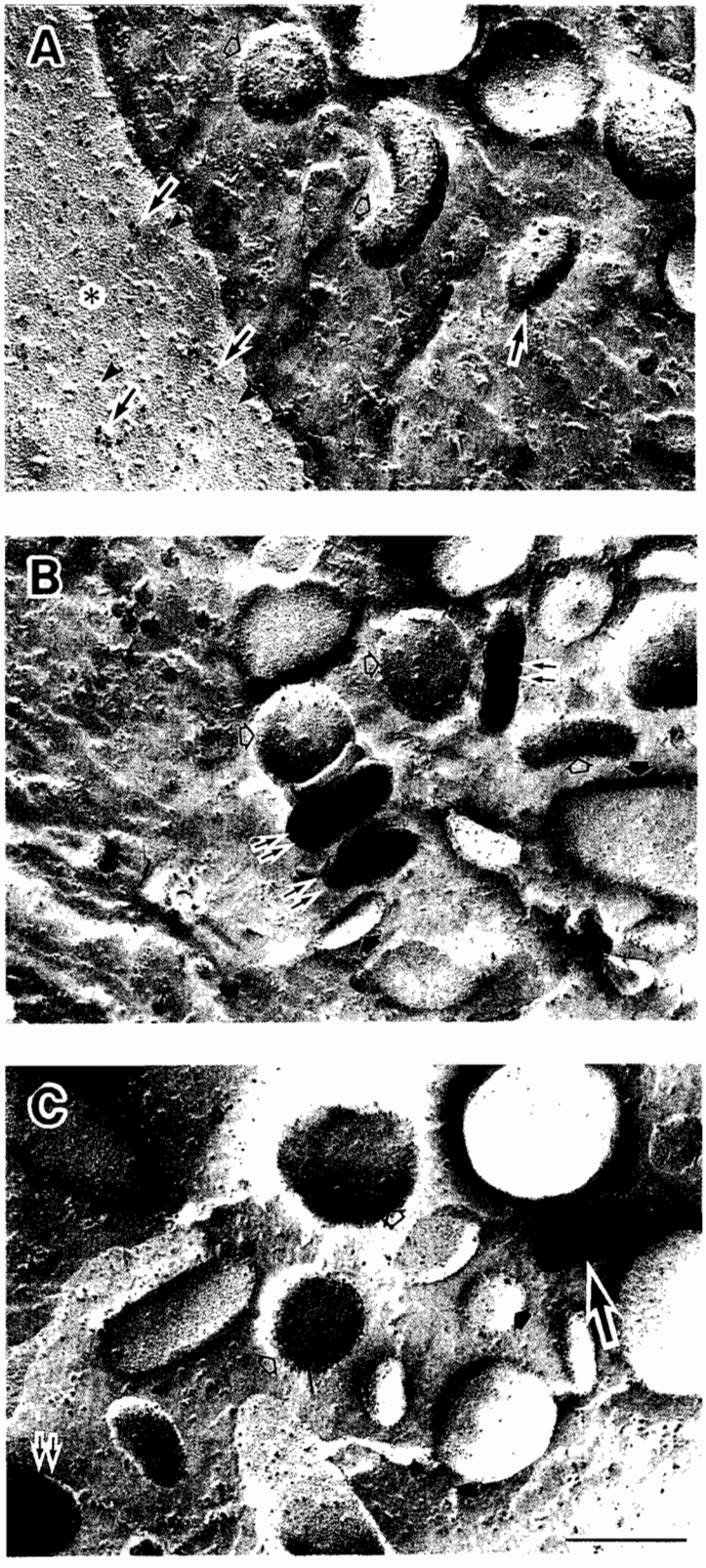

Fig. 2. Topology of glycosyl-phosphatidylinositol (GPI)-anchored proteins in unstimulated human neutrophils prepared by OG-digestion fracture-labeling. **A.** Immunocytochemical labeling of CD16. Immunogold particles (10-nm colloidal gold) showing CD16 (large arrows) are present both on the exoplasmic half (E-face) of the plasma membrane (*) and on that of a cytoplasmic granule in a resting cell. Intramembrane particles (IMPs) (arrowheads) are evident and distinct from colloidal gold particles. E-faces (open arrows) and protoplasmic halves (P-faces) (small arrows) of other types of intracellular granules are evident. **B.** Enzyme cytochemical labeling of alkaline phosphatase (ALPase). The electron-dense reaction product showing ALPase activity is localized on the E-faces of small intracellular granules (double arrows). **C.** Double labeling of CD16 and ALPase. Immunogold particles showing CD16 are present on the E-face of the granule that is labeled with cerium demonstrating ALPase (large arrow). A granule labeled with cerium alone is also present (double arrow). Note that co-localization of CD16 and ALPase labeled with different cytochemical probes is readily recognized in the same granule. [Panel C reprinted from Takizawa et al. (1998) with permission]. Bar: 0.2 μm.

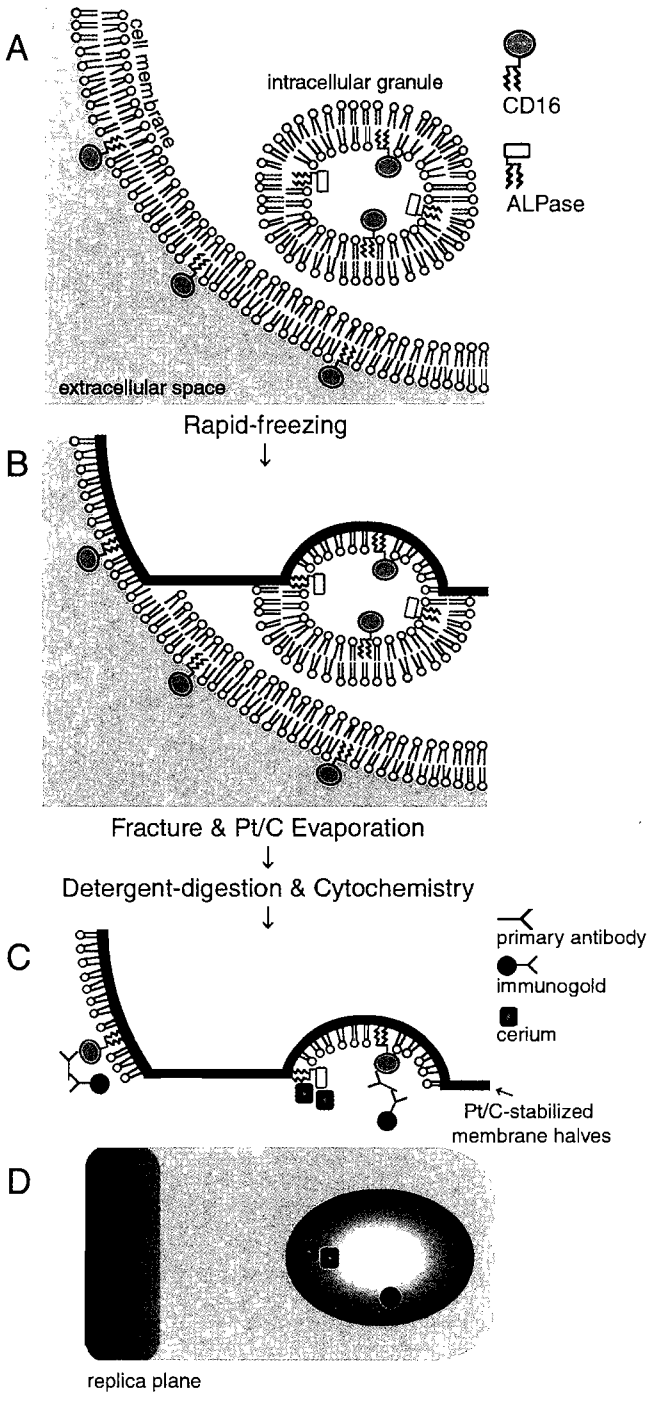

Fig. 3. Diagrammatic representation of the detergent-digestion fracture-labeling of GPI-anchored proteins. Unfixed cell is rapidly frozen **(A)**, freeze-fractured, and then physically stabilized with evaporated Pt/C **(B)**. Cell is digested with OG and labeled by immunocytochemistry and subsequent enzyme cytochemistry **(C)**. The Pt/C-stabilized membrane halves are labeled with two different cytochemical markers; in this case CD16 molecules are detected with immunogold particles while an ALPase molecule is demonstrated with cerium reaction product. **D.** Simulated electron microscopic image of the cytochemically labeled replica shown in panel C. These cytochemical markers are superimposed on the panoramic view of the replica.

influence on enzyme cytochemical detection than on immunocytochemical detection in this method. We have recently shown that detection of enzyme activity on replicas becomes difficult when the concentration of OG exceeds 20 mM, whereas such concentrations did not affect the immunocytochemical detection of proteins (Takizawa et al., 1998). We therefore recommend that OG be used at its CMC (~20 mM) in fracture-labeling methods.

In the double labeling technique, the order of cytochemical reactions is critical; immunocytochemical localization should always be the first step in order to eliminate a possible interference by enzyme cytochemical reactions. Enzyme cytochemical reaction products (i.e., heavy metal depositions) may modify the efficiency of the of immunocytochemical detection. In application of this combined approach, control experiments using quantitative analysis of immunogold labeling as well as conventional cytochemical controls are necessary to determine whether the particular enzyme cytochemical reaction affects immunocytochemical labeling.

Applications of detergent-digestion fracture-labeling technique

We have used human neutrophils as the model cell system to examine the versatility of the OG-digestion fracture-labeling technique and examined the subcellular distribution of certain glycosyl-phosphatidylinositol (GPI)-anchored proteins. In human neutrophils, some proteins, such as CD16 (FcRIIIB) and alkaline phosphatase (ALPase), are covalently attached to GPI in the exoplasmic membrane halves of the plasma membrane and a subset of intracellular granule membranes (Fig. 3). These proteins are expressed at relatively low levels and stored in small intracellular granules in unstimulated cells; they can be rapidly up-regulated to the cell-surface in a stimulus-dependent fashion (Kobayashi and Robinson, 1991; Cain et al., 1995). Since neutrophils contain abundant intracellular granules, it is often difficult to identify GPI-anchored protein-containing granules from other granules without the use of cytochemical markers.

I. Immunocytochemical labeling of GPI-anchored proteins in human neutrophils

For immunocytochemical fracture-labeling, OG efficiently digested unfractured cell components and adequately retained immunocytochemical antigenicity on human neutrophil replicas. CD16 was present both in plasma membranes and in small cytoplasmic granules in the cells (Fig. 2A).

There is no apparent difference in fracture faces of replicas prepared by the detergent-digestion fracture-labeling technique when compared to those processed by conventional freeze-fracture electron microscopy. Since split membrane halves are electron-lucent and physically

"fixed" with Pt/C replicas, immunogold particles indicating distribution of specific antigens on the split membranes are superimposed on the view of the replicas under electron microscopic examination (Fig. 3). Thus, we described the immunocytochemical distribution of CD16 as follows: CD16 was detected both on the E-face of plasma membranes and on that of small intracellular granules in neutrophils (i.e., CD16 was located both on the outer surface of cell membranes and on the inner surface of intracellular granule membranes) (Figs. 2A, 3).

II. Enzyme cytochemical labeling of GPI-anchored proteins in human neutrophils

As mentioned above, the OG-digestion fracture-labeling technique preserves the biological activity of enzyme molecules on Pt/C-stabilized membrane halves, thereby revealing ALPase-positive granule membranes on the replicas of neutrophils. In this case, ALPase was labeled with the enzyme cytochemical marker cerium (Robinson and Karnovsky, 1983; Kobayashi and Robinson, 1991). The method is based on the principle that phosphate ions are liberated from a suitable substrate (e.g., ß-glycerophosphate) (Fig. 4). The enzyme-generated phosphate ions are trapped at the site of formation by cerium present in the reaction medium forming highly insoluble cerium phosphate, which appears as an electron-dense deposits (Fig. 4). ALPase was found predominantly on the freeze-fractured membranes of small cytoplasmic granules (Fig. 2B). In ALPase-containing granules, the E-face of the small granule membranes was preferentially labeled with the cerium phosphate reaction product (Figs. 2B, 3).

III. Double labeling of GPI-anchored proteins in human neutrophils

Double labeling of CD16 and ALPase was also achieved on the same replicas prepared by the OG-digestion fracture-labeling technique (Fig. 2C). In unstimulated cells, CD16 was present on both the E-face of plasma membranes and that of small cytoplasmic granules. Immunogold particles demonstrated that CD16 molecules were also present on the E-faces of granules labeled with reaction product demonstrating ALPase. It is noteworthy that since the electron density of immunogold particles was distinct from that of the cerium phosphate reaction product, co-localization of two different proteins labeled with different cytochemical probes was readily distinguishable (Figs. 2C, 3). The results that a relatively small amount of these proteins remain attached to the Pt/C replicas via GPIs in the membrane halves lend support to the contention that physical "fixation" of the fractured membranes by Pt/C evaporation prevents the extraction of membrane constituents in those fracture planes during detergent-digestion. OG-digestion is important for the success of combined cytochemistry on replicas. It is also emphasized that the detergent-digestion fracture-labeling technique can reveal biochemical properties of each intracellular membrane system *in situ*.

The potential for membrane protein partitioning is an important consideration for cytochemical labeling of replicas. Fujimoto (1997) discussed various problems related to partition in immunocytochemical labeling of replicas. We have also discussed similar problems encountered in enzyme cytochemical labeling of replicas (Takizawa and Saito, 1997). Therefore, this topic is not considered herein.

Ultrasmall immunogold labeling of replicas

Colloidal gold particles of 10- to 15-nm in diameter have been used routinely as a secondary detection system in the detergent-digestion fracture-labeling technique, because smaller particles (e.g., 5-nm) are not readily identified on the somewhat granular Pt/C-replicas. However labeling of replicas using larger-sized gold particles is problematic since these particles are less efficient for labeling than the smaller particles. The poor

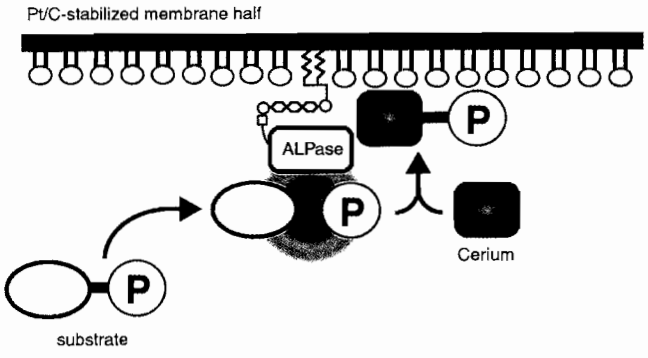

Fig. 4. Cerium based enzyme cytochemistry of ALPase on replicas. Inorganic phosphate (P) is released from a substrate (i.e., ß-glycerophosphate) in a reaction medium by enzymatic reaction of an ALPase molecule on the membrane half. The phosphate ion is trapped at the site of formation by cerium present in the reaction medium, and is deposited as highly insoluble cerium phosphate.

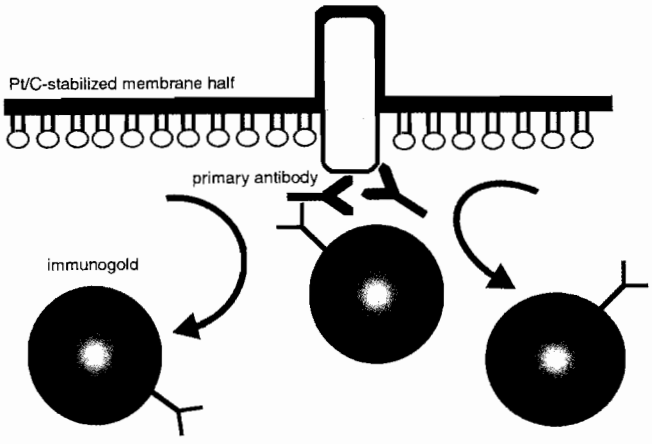

Fig. 5. Schematic illustration depicting possible steric hindrance by larger-sized immunogold in immunocytochemical labeling of replicas.

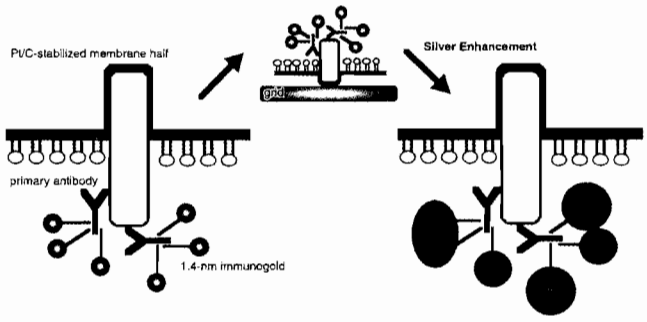

Fig. 6. Diagrammatic representation of ultrasmall gold immunocytochemistry on replicas. In this case, 1.4-nm immunogold is used as the secondary antibody instead of colloidal gold immunoprobes. Immunostained membrane half is mounted on a formvar-coated nickel grid with its Pt/C cast in contact with the thin formvar film, dried, and then silver-enhanced to visualize the ultrasmall gold particles by conventional electron microscopy.

labeling is mainly due to the size of gold particle (e.g., steric hindrance effects) (Fig. 5).

An alternative approach is to use ultra-small immunogold probes (≈ 1-nm). One such ultra-small immunogold probe is Nanogold™ which consists of 1.4-nm gold particle covalently conjugated with an affinity-purified Fab' fragment (for review see Robinson et al., 1998). These 1.4-nm immunogold probes have been introduced to freeze-fracture immunocytochemistry (Takizawa, 1999). In this case, Nanogold is employed as a secondary antibody instead of colloidal gold immunoprobes. Silver enhancement is required to visualize the ultrasmall gold particles because it is difficult to detect these particles against the background of the Pt/C replica by conventional electron microscopy. After OG-digestion fracture-labeling using Nanogold, immunostained membrane halves were mounted on formvar-coated nickel grids with their Pt/C cast in contact with the thin formvar film (i.e., the membrane halves were exposed), dried, and then silver-enhanced using the method of Burry (1995) (Fig. 6).

We used the detergent-digestion fracture-labeling technique with 1.4-nm immunogold particles to examine the two-dimensional distribution of HLA class I antigens on the replicas of human neutrophils (Fig. 7). The use of ultrasmall (1.4-nm) gold immunoprobes yielded heavier gold-labeling of HLA class I in the replicas than did 10-nm colloidal gold particles (Fig. 7). Furthermore, by comparing the labeling density of different-sized immunogold probes reflecting the distribution of HLA class I, we demonstrated that the distribution density was inversely related to the size of gold particles (i.e., 1.4-nm > 5-nm > 10-nm > 15-nm) (Takizawa, 1999). In addition, our study also revealed that Pt/C casts associated with split membrane halves were neither degraded nor themselves enhanced by the silver enhancement method; all electron-dense particles on the replicas were silver-enhanced ultrasmall gold particles. Hence, the 1.4-nm immunogold probe in conjugation with silver enhancement is a valuable addition for the detergent-digestion fracture-labeling technique.

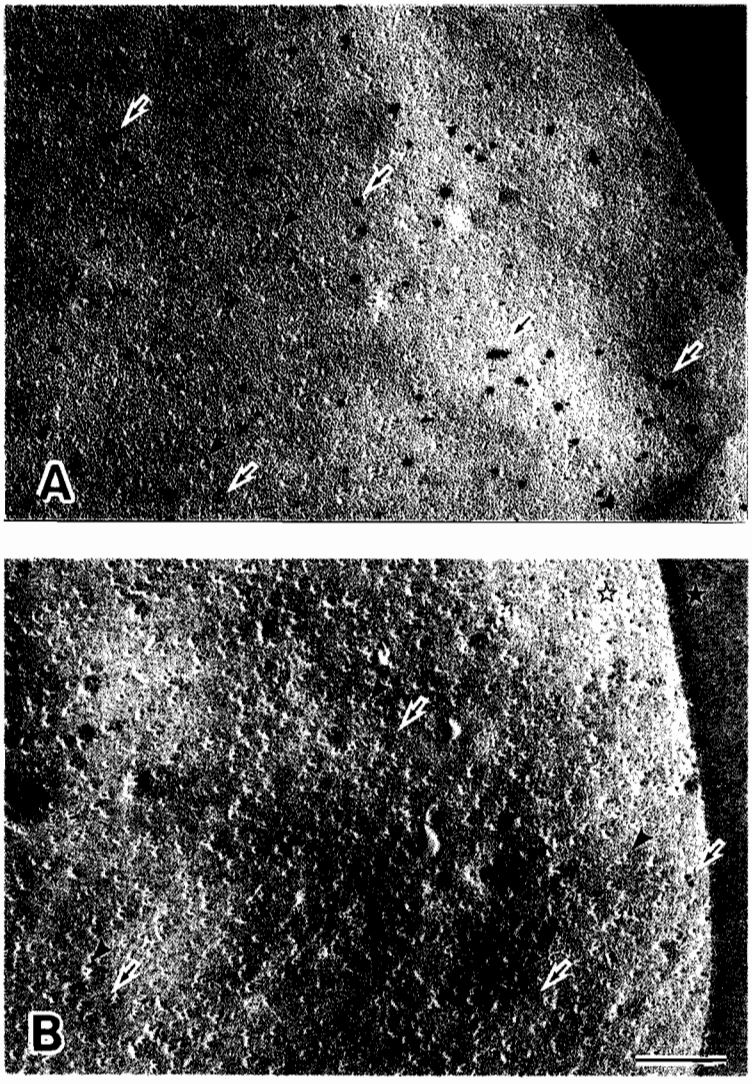

Fig. 7. Immunocytochemical labeling of HLA class I in human neutrophil replicas. **A.** Electron micrograph of a plasma membrane replica showing the distribution of HLA class I achieved using 1.4-nm gold-conjugated secondary antibody and subsequent silver enhancement. Note the silver-enhanced 1.4-nm gold particles (arrows) on the E-face of the plasma membrane (open star). Note also the presence of IMPs (arrowheads). Freeze-fractured extracellular space (filled star) is evident. **B.** Electron micrograph of a plasma membrane replica indicating HLA class I achieved using 10-nm gold-conjugated (arrows). Note the difference in gold-labeling density between the 1.4-nm gold-labeled replica (A) and 10-nm gold-labeled replica (B). Bar: 0.2 μm.

Conclusions

Freeze-fracture cytochemistry allows visualization of cellular and molecular features of biomembranes *in situ*. Freeze-fracture cytochemistry has been improved by numerous technical advancements and augmented by improved cytochemical detection systems. In this review, we discuss the detergent-digestion fracture-labeling technique. We show the OG-digestion fracture-labeling technique as a versatile tool for direct analysis of the macromolecular architecture of biological membranes and identification of particular intracellular membrane organelles. In addition, we reviewed the application of ultrasmall gold to freeze-fracture immunocytochemistry. Freeze-fracture cytochemistry is a valuable technique for the study of topology and dynamics of membrane molecules.

Acknowledgements. We are grateful to Dr Takuma Saito, Dr Takashi Yashiro, and Dr Shigeo Ookawara for their support and encouragement. We also thank Ms Kiyomi Inose of the Campus Microscopy and Imaging System at Jichi Medical School, Ms Megumi Yatabe, and Ms Michiyo Soutome for their assistance. This work was supported by grants-in-aid for Scientific Research and project grants of Center for Molecular Medicine at Jichi Medical School from the Ministry of Education, Science, Sports, and Culture of Japan (TT), and by NIH grant HD35121 and the American Heart Association, Ohio Valley Affiliate (JMR).

References

Baron C. and Thompson T.E. (1975). Solubilization of bacterial membrane proteins using alkyl glucosides and dioctanoyl phosphatidylcholine. Biochim. Biophys. Acta 382, 276-285.

Burry R.W. (1995). Pre-embedding immunocytochemistry with silver enhanced small gold particles. In: Immunogold-silver staining. Principles, methods and applications. Hayat M.A. (ed). CRC Press. Boca Raton. pp 217-230.

Cain T.J., Liu Y., Takizawa T. and Robinson J.M. (1995). Solubilization of glycosyl-phosphatidylinositol-anchored proteins in quiescent and stimulated neutrophils. Biochim. Biophys. Acta 1235, 69-78.

Carpentier J.L., Brown D., Iacopetta B. and Orci L. (1985). Detection of surface-bound ligands by freeze-fracture autoradiography. J. Cell Biol. 101, 887-890.

Elias P.M., Friend D.S. and Goerke J. (1979). Membrane sterol heterogeneity: freeze-fracture detection with saponins and filipin. J. Histochem. Cytochem. 27, 1247-1260.

Fisher K.A. and Branton D. (1976). Freeze-fracture autoradiography: feasibility. J. Cell Biol. 70, 453-458.

Fujimoto K. (1995). Freeze-fracture replica electron microscopy combined with SDS digestion for cytochemical labeling of integral membrane proteins: application to the immunogold labeling of intercellular junctional complexes. J. Cell Sci. 108, 3443-3449.

Fujimoto K. (1997). SDS-digested freeze-fracture replica labeling electron microscopy to study the two-dimensional distribution of integral membrane proteins and phospholipids in biomembranes: practical procedure, interpretation and application. Histochem. Cell Biol. 107, 87-96.

Fujimoto K. and Pinto da Silva P. (1992). Fracture-flip/Triton X-100 reveals the cytoplasmic surface of human erythrocyte membranes. Acta Histochem. Cytochem. 25, 255-263.

Fujimoto K., Umeda M. and Fujimoto T. (1996). Transmembrane phospholipid distribution revealed by freeze-fracture replica labeling. J. Cell Sci. 109, 2453-2460.

Gould R.J., Ginsberg B.H. and Spector A.A. (1981). Effect of octyl ß-glucoside on insulin binding to solubilized membrane receptors. Biochemistry 20, 6776-6781.

Hall C.E. (1950). A low temperature replica method for electron microscopy. J. Appl. Phys. 21, 61-62.

Helenius A. and Simons K. (1975). Solubilization of membranes by detergents. Biochim. Biophys. Acta 415, 29-79.

Kobayashi T. and Robinson J.M. (1991). A novel intracellular compartment with unusual secretory properties in human neutrophils. J. Cell Biol. 113, 743-756.

Pinto da Silva P. (1987). Topology, dynamics, and molecular cytochemistry of integral membrane proteins: a freeze-fracture view. In: Electron microscopy of proteins. Membranous structures. Vol. 6. Harris J.R. and Horne R.W. (eds). Academic Press. London. pp 1-38.

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

Pinto da Silva P., Kachar B., Torrisi M.R., Brown C. and Parkison C. (1981a). Freeze-fracture cytochemistry: replicas of critical point-dried cells and tissues after fracture-label. Science 213, 230-233

Pinto da Silva P., Parkison C. and Dwyer N. (1981b). Freeze-fracture cytochemistry: thin sections of cells and tissues after labeling of fracture faces. J. Histochem. Cytochem. 29, 917-928.

Pinto da Silva P., Torrisi M.R. and Kachar B. (1981c). Freeze-fracture cytochemistry: localization of wheat-germ agglutinin and concanavalin A binding sites on freeze-fractured pancreatic cells. J. Cell Biol. 91, 361-372.

Rix E., Schiller A. and Taugner R. (1976). Freeze-fracture-autoradiography. Histochemistry 50, 91-101.

Robinson J.M. and Karnovsky M.J. (1983). Ultrastructural localization of several phosphatases with cerium. J. Histochem. Cytochem. 31, 1197-1208.

Robinson J.M., Takizawa T., Vandré D.D. and Burry R.W. (1998). Ultrasmall immunogold particles: important probes for immunocytochemistry. Microsc. Res. Tech. 42, 13-23.

Severs N.J. (1991). Freeze-fracture cytochemistry: a simplified guide and update on developments. J. Microsc. 161, 109-134.

Shinoda K., Yamaguchi T. and Hori R. (1961). The surface tension and the critical micelle concentration in aqueous solution of ß-D-alkyl glucosides and their mixtures. Bull. Chem. Soc. Japan 34, 237-241.

Slot J.W. and Geuze H.J. (1981). Sizing of protein A-colloidal gold probes for immunoelectron microscopy. J. Cell Biol. 90, 533-536.

Takizawa T. (1999). High-resolution immunocytochemical labeling of replicas with ultrasmall gold. J. Histochem. Cytochem. 47, 569-573.

Takizawa T., Nakazawa E. and Saito T. (1997). Freeze-fracture enzyme cytochemistry reveals the distribution of enzymes in biological membranes: enzyme cytochemical label-fracture and fracture-label. Acta Histochem. Cytochem. 30, 77-84.

Takizawa T. and Saito T. (1996). Freeze-fracture enzyme cytochemistry: application of enzyme cytochemistry to freeze-fracture cytochemistry. J. Electron Microsc. 45, 242-246.

Takizawa T. and Saito T. (1997). New fracture-labelling method: alkaline phosphatase in unstimulated human neutrophils. J. Electron

Microsc. 46, 85-92.

Takizawa T., Saito T. and Robinson J.M. (1998). Freeze-fracture cytochemistry: a new method combining immunocytochemistry and enzyme cytochemistry on replicas. J. Histochem. Cytochem. 46, 11-17.

Torrisi M.R. and Mancini P. (1996). Freeze-fracture immunogold labeling. Histochem. Cell Biol. 106, 19-30.

Verkleij A.J., Kruijff B. de, Gerritsen W.F., Demel R.A., Deenen L.L. van and Ververgaert P.H. (1973). Freeze-etch electron microscopy of erythrocytes, *Acholeplasma laidlawii* cells and liposomal membranes after the action of filipin and amphotericin B. Biochim. Biophys. Acta 291, 577-581.

Accepted September 8, 1999