Scanning Electron Microscopy

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

Article 10

1985

Complementary Replicas of Ultra-Rapidly Frozen Specimens

M. J. Costello
Duke University

R. D. Fetter
Duke University

T. G. Frey
University of Pennsylvania, Philadelphia

Science of Biological Specimen Preparation (pp. 95-101)
SEM Inc., AMF O'Hare (Chicago), IL 60666-0507, U.S.A.

COMPLEMENTARY REPLICAS OF ULTRA-RAPIDLY FROZEN SPECIMENS

M.J. Costello*, R.D. Fetter and T.G. Frey+

Department of Anatomy, Duke University Medical Center, Durham, NC 27710 and +Department of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, PA 19104

Abstract

Complementary freeze-fracture replicas have been prepared of ultra-rapidly cooled specimens in the absence of chemical pretreatments. The grid-sized replicas were stabilized by open mesh gold grids during the cleaning process and, after cleaning, were supported on thin Formvar films. The complementary replicas were valuable for describing artifacts, for interpreting the nature of fracture planes and for evaluating the resolution of replicas. Complementary images demonstrated that heat emitted from resistance electrodes or electron guns during evaporation can seriously distort fracture surfaces even for samples held at -150°C. Complementary images of crystalline membranous cytochrome c oxidase helped establish that fracturing reveals hydrophilic surfaces. Complementary images of proteoliposomes showed that intramembrane particles produced by an integral 140kdal protein generated complementary pits whereas intramembrane particles produced by a smaller integral 46.5kdal protein did not. The inability to observe the small pits is in part a limitation of the resolution of conventionally prepared platinum/carbon replicas.

Key Words: Freeze-fracture, complementary replicas, crystalline membranes, proteoliposomes, heat damage, intramembrane particles, hydrophilic fractures, cytochrome o oxidase, lac permease

*Address for correspondence:

M.J. Costello
Department of Anatomy
Box 3011
Duke University Medical Center
Durham, NC 27710
Phone no.: (919) 684-4124

Introduction

Complementary images of freeze-cleaved and replicated biological specimens provide unique opportunities to evaluate the quality of the replicas. Complementary replicas are valuable for determining (a) the effect of surface heating during metal evaporation, (b) the extent of plastic deformation, (c) the amount of contamination, (d) the resolution limit of the metal coating, and (e) the location and nature of membrane fracture faces.

Complementary replicas first presented by Steere and Moseley (1969) and subsequently by several laboratories in the 1970s (see literature summary in Fetter and Costello, 1986) were obtained most commonly by using either a plastic film to stabilize the replicas (Steere, 1973) or a pair of gold grids sandwiched with the samples which when split apart would support the complementary replicas (Muehlethaler et al., 1970). Nearly all of the early preparations employed relatively large cryoprotected specimens frozen conventionally in liquid coolants. We have developed procedures which employ a gold grid to stabilize the replicas and permit the recovery of intact grid-sized replicas from ultra-rapidly cooled thin specimens sandwiched between copper sheets (Costello, 1980; Costello et al., 1984a; Fetter and Costello, 1986). Examples of complementary replicas presented here illustrate some of the factors which limit the resolution and image quality of conventional platinum/carbon replicas.

Materials and Methods

Specimens were sandwiched between copper strips and ultra-rapidly cooled in liquid propane as described previously (Costello,1980; Costello et al., 1984a). Sandwiches were fractured in double replica devices (Costello et al., 1984a; Gross et al.,1984). Fractured surfaces were shadowed using either resistance electrodes in a Balzers BA360 or electron guns in a Balzers BA400. The evaporation settings used were those suggested by the manufacturer with one exception. The power supply of the Balzers BA360 was converted to direct current based on the findings of Watt (1974) that direct current reduced the

heat output of the source. Samples were equilibrated from -150°C to -180°C. Immediately after fracturing, 1-2nm platinum/carbon was deposited at an inclination of 45°. Samples were backed with carbon from 90°.

Replicas were recovered as described in detail in Fetter and Costello (1986) and Costello and Fetter (1986). In brief summary, copper strips which contain the replicated samples on the $(2.5mm)^2$ plateaus are transferred from the freeze-fracture device to wells in a porcelain depression plate. A drop of chromic acid is spread over the wings of the copper strip (Fig. 1A) and then the level of acid is raised to float the strip so that the wings are submerged and the plateau remains dry and above the surface (Fig. 1B). A 50-mesh gold grid (PELCO, cat. no. 1GG50) is placed on top of the replica just after the copper wings fall off; the grid protects the replica during the cleaning process. The grid has a marker which is aligned along the long axis of the copper strip and facilitates the later matching of complememtary regions. Immediately after the copper of the plateau dissolves, the acid is changed by lowering the level of liquid in the well with a micropipette connected to a weak vacuum line (Fig. 1C). The chromic acid is removed with several water washes. To digest the sample, bleach is introduced in gradually increasing concentration to a maximum of 70%. After the sample is digested (usually within 2h), the replica/grid units are washed several times with distilled water. The distilled water is conveniently introduced through a fine micropipette. Each well is drained or filled within 10-20sec without agitation of the replica. Cleaned replica/grid units are picked up from below with thin Formvar films suspended in platinum wire loops (Fig. 1D). After about an hour, the films are sufficiently dry to remove the gold grid by breaking away the excess Formvar film from around the grid with a sharp object. Low magnification montages are made (Fetter and Costello, 1986) and complementary regions located; the central markers on the gold grids are used as guides.

Complementary images were obtained on Philips 301 or 420 microscopes and are mounted in positive contrast with the dividing line either horizontal or vertical. The shadow direction is perpendicular to the dividing line for the Balzers BA360 (Figs. 2-4) and is parallel to the dividing line for the Balzers BA400 (Fig. 5).

Results

The influence of surface heating during metal evaporation.

Heat deposited during metal replication comes from three sources (a) the kinetic energy of the metal atoms and clusters, (b) electrons and ions and (c) radiant (photon) energy. For resistance or electron gun evaporators the greatest source of heating is probably the radiant energy (Abermann et al., 1970).

The heat reaching the sample surfaces during metal evaporation affects the appearance of fracture faces in several ways. Before a layer of metal sufficiently thick to stabilize the frozen surface is deposited, the heat from the source can raise the surface temperature enough to cause sublimation of ice (etching). The amount of sublimation depends on a number of factors which include the local vacuum and the distance of the exposed surface from the highly conductive copper support. When exposed surfaces are not affected uniformly, the heating can be detected in complementary images, as for a suspension of membranes from the mammalian lens (Zampighi et al., 1982) in Fig. 2. In Fig. 2A the circular profile of one membrane vesicle (arrow) is barely visible whereas in Fig. 2B etching reveals the profile clearly (arrow). Etching has also accentuated the ice crystals in the background which appear as undulations in Fig. 2B, indicating that this region of the specimen was not frozen very rapidly.

An additional effect of surface heating is the deformation of structures which extend above fracture planes. The complementary images in Fig. 3 of a vesicle crystal of mitochondrial membranous cytochrome c oxidase (Frey et al., 1982) demonstrate that surface heating can deform and flatten protruding structures. In Fig. 3B the crystalline lattice is disrupted (large arrow) by the formation of jagged troughs during fracturing. The complementary image should contain complementary ridges, but Fig. 3A shows instead a relatively smooth plateau in which the lattice rows are faintly visible (large arrow). Other structures which extend above the crystalline fracture plane appear to have rounded and smooth surfaces, such as the mound in Fig. 3B (small arrow), a form of contamination which is probably a fragment released at time of fracture since no corresponding pit is observed in Fig. 3A.

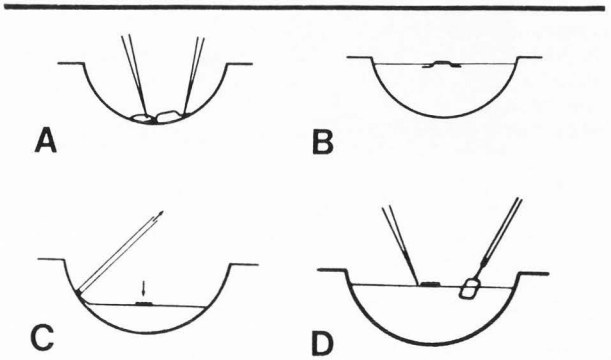


Figure 1. Replica recovery. (A) A drop of chromic acid is spread over the wings of a copper strip. (B) A copper strip floating on chromic acid. A gold grid is placed on the plateau just after the wings fall off. (C) A micropipette tip is held against the side of a well to withdraw fluid with minimum turbulence. A similar procedure is used to introduce fluids. (D) A sharp object is used to nudge the cleaned replica/grid unit up to a thin Formvar film supported in a wire loop which is used to pick up the replica/grid unit. [Reprinted with permission from M.J. Costello and R.D. Fetter, Methods Enzymol. 127, 704-718 (1986).]

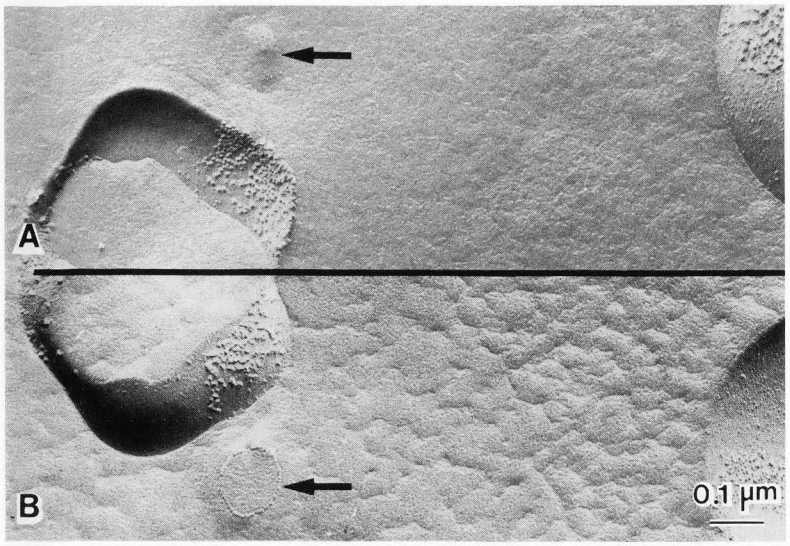


Figure 2. Complementary images of a suspension of membranes from the mammalian lens. The heat deposited during evaporation has caused etching of B which reveals a circular membrane profile that is only faintly visible in A. [Reprinted with permission from M.J. Costello and R.D. Fetter, Methods Enzymol. 127, 704-718 (1986).]

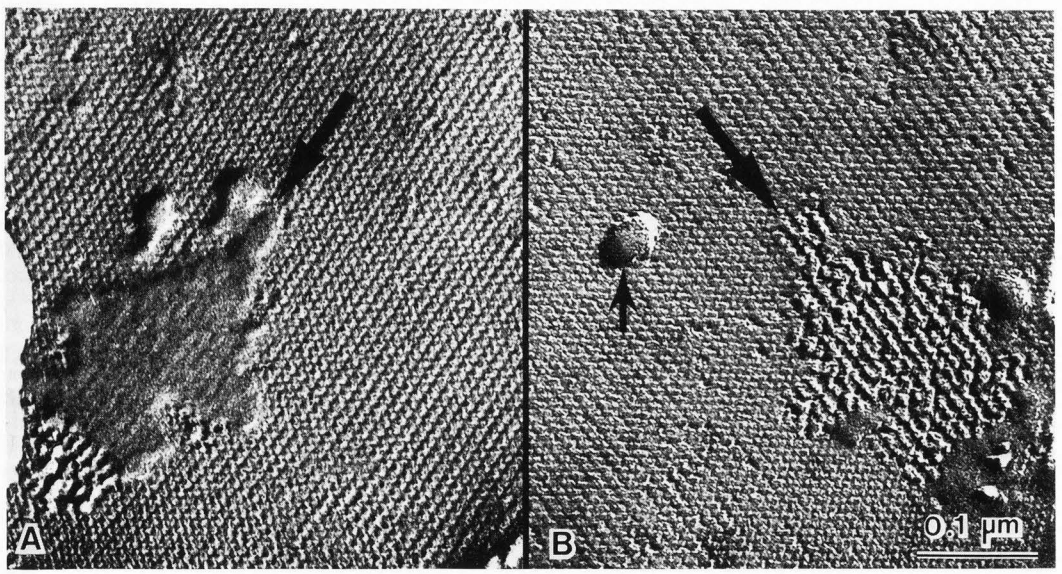
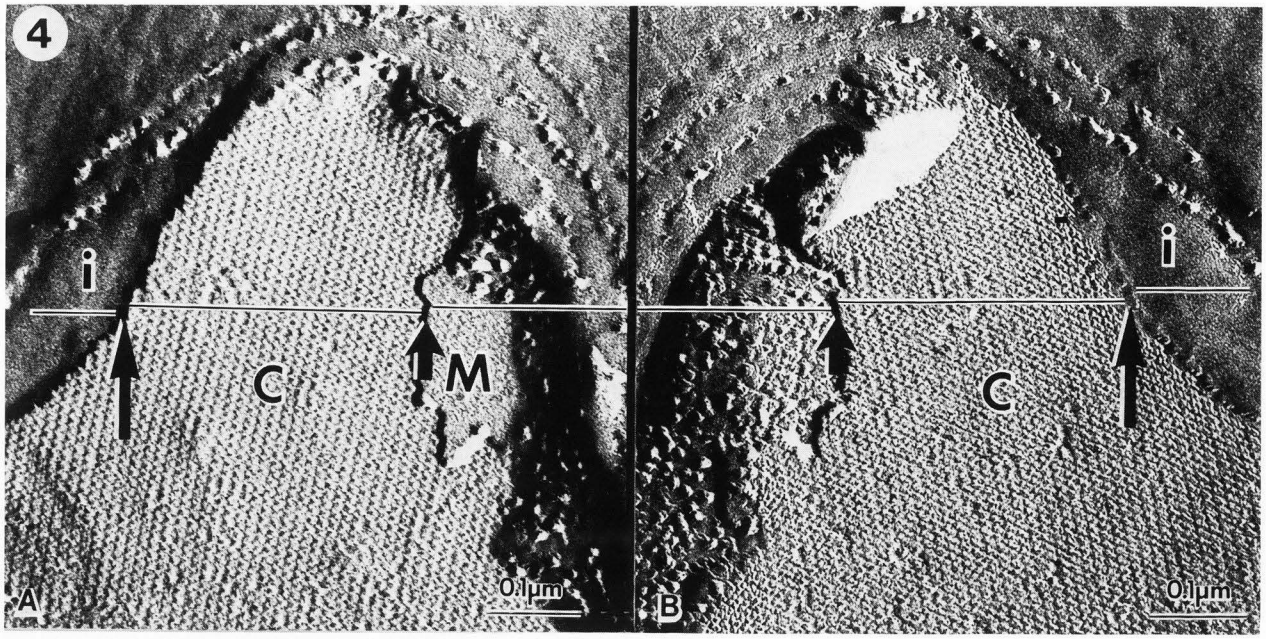
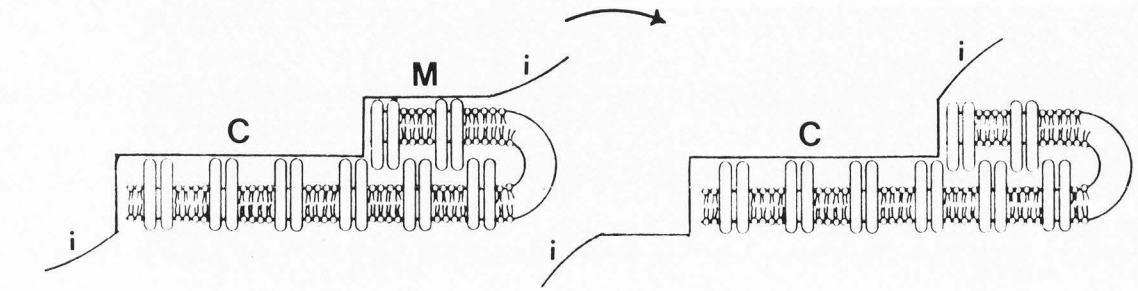


Figure 3. Complementary images of a vesicle crystal of mitochondrial membranous cytochrome c oxidase. The crystalline areas show almost perfect complementarity. Evidence for surface heating during evaporation is seen in the plateau in A (large arrow) because this region should display jagged ridges which match the jagged troughs in the complementary region in B (large arrow). The material pulled from B during fracturing appears to have been melted during metal deposition. The large mound in B (small arrow) is probably contamination.

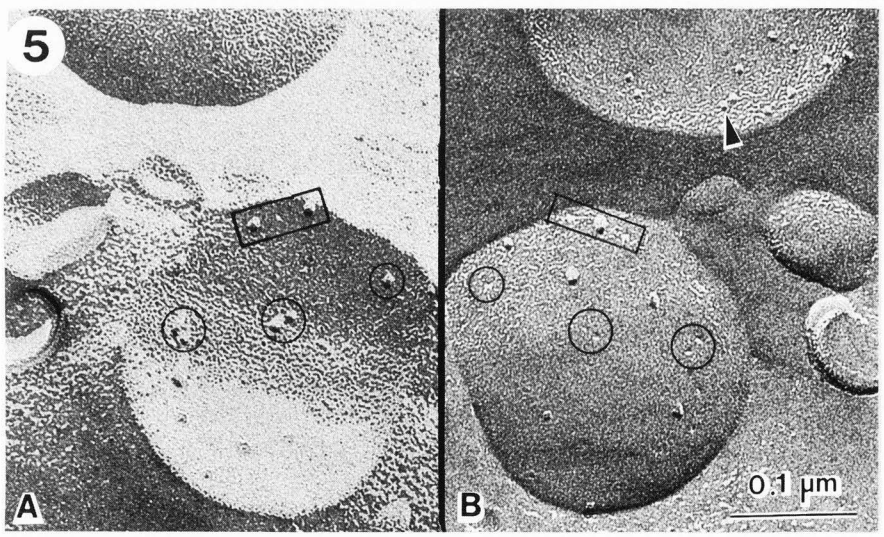
Fracture plane location in complementary replicas.

Although complementary images cannot by themselves provide a unique determination of the location of fracture planes, they can be used to test the self-consistency of a model for a fracture pattern. As an example, another region of the same vesicle crystal in Fig. 3 is shown in Fig. 4 to illustrate the relationship of the fracture planes to the surrounding ice (i). The large crystalline surfaces (C) in Fig. 4A and B are separated from ice by steps (large arrows) which are approximately the same height as the steps (small arrows) to the small crystalline surface (M). This pattern is consistent with the model shown in Fig. 4C in which hydrophilic surfaces are exposed as proposed previously (Costello and Frey, 1982). Such an unusual fracture pattern appears to occur only in regions which contain the highly crystalline array of proteins. In regions of membrane where there is no crystalline array, the classic bilayer fracture proposed by Branton (1966) is observed (Costello and Frey, 1982).





C



Resolution limit of metal replicas.
One measure of the resolution of a metal replica is the visibility of small structures of approximately known dimensions. As the quality of a replica improves, smaller structures will become visible. This principle is illustrated in

Figure 4. Complementary images (A and B) and fracture model (C) of the same cytochrome c oxidase crystalline membranes displayed in Fig. 3. Fracture steps (large arrows) from ice (i) to large surface "C" are approximately the same height as those from surfaces "C" to "M" (small arrows). The schematic diagram explains the observed fracture pattern in terms of hydrophilic fracture faces (see Costello and Frey, 1982).

Figure 5. Complementary images of reconstituted proteoliposomes of lac permease and cytochrome o oxidase in approximately 1:1 molar ratio. The large 9nm diam. intramembrane particles, produced by the 140kdal cytochrome o oxidase, have complementary pits (circles and rectangle). The small 7nm diam. intramembrane particles (arrowhead), produced by the 46.5kdal lac permease, do not have matching pits.

Fig. 5 for the mixed reconstituted proteoliposomes containing two distinct integral membrane transport proteins, lac permease and cytochrome o oxidase, isolated from E. coli and reconstituted into E. coli phospholipids (Matsushita et al., 1983). Functional and biochemical assays have established that both proteins are transmembrane and both generate intramembrane particles in fracture planes (cf. review Kaback, 1985). The larger protein, cytochrome o oxidase, 140kdal, produces particles about 9.0nm diam. (Matsushita et al., 1984) and complementary pits (Fig. 5 circles and rectangle). The smaller lac permease, 46.5kdal, produces 7.0nm dia. particles (arrowhead; Costello et al., 1984b) but no corresponding pits can be detected. Because lac permease pits should be present, it is proposed that the large grain size of the platinum/carbon replicas (approx. 2.5-3.0nm) does not permit the visualization of the small pits.

Discussion

Procedures have been developed for the routine recovery of complementary replicas of ultra-rapidly cooled specimens sandwiched between thin copper sheets (Fetter and Costello, 1986). The high yield of intact replicas (approx. 80% in our laboratory) and the ease of locating complementary regions within the replicas has made possible the examination of some of the factors which affect replica quality.

The quality of platinum/carbon replicas of freeze-cleaved specimens is influenced by the heat deposited from the evaporation source. Although the bulk of a sample may remain at low temperature during the replication step, the temperature of the superficial layer may rise sufficiently to promote the sublimation of ice (Fig. 2). A rough estimation of the increase in surface temperature can be made from the relationship of sublimation rate to surface temperature at a given vacuum (Davy and Branton, 1970). At a vacuum of 10^{-6} torr, an initial temperature of -150°C and about 2 sec of exposure to the evaporation source before a protective metal layer is formed (the conditions applicable to the images in Fig. 2), a surface temperature of about -90°C could account for the etching of about 30 nm of water. It should be emphasized that the estimated 60°C rise in surface temperature is a very rough approximation but is consistent with the thermocouple measurements of Belous and Wayman (1967) who observed temperature increases of 100-500°C under somewhat different shadowing conditions. It is likely that the amount of surface heating is highly variable and depends on the composition of the sample, the local shadow inclination angle of the surfaces to the source, the proximity of the surface to a heat sink (copper support) and the properties of the evaporation source. A low initial temperature of the sample should reduce the effects of surface heating. Gross et al. (1985) found that sample temperatures near -260°C, as well as vacuums in the 10^{-9} torr range, were instrumental in producing fine grain, high quality platinum/carbon and tantalum/tungsten replicas. The heat from the source can be reduced with shuttering; for example, Ellisman and Staehelin (1979) used a shutter to protect the samples from the warm-up period of the source and Gulik et al. (1982) exposed samples intermittently to a tantalum/tungsten source to reduce heat damage. Ultimately, all forms of extraneous energy from the source can be eliminated with a velocity selector (Aldrian and Horn, 1974); however, such a device has not yet been successfully applied to freeze-fracture experiments.

The heat-induced deformation of the fractured surface in Fig. 3 is obvious because of the unusual fracture pattern (Fig. 4) and because of the proximity of the crystalline lattice which is relatively unaffected by the deposited heat. The heat-induced deformation of integral proteins which form intramembrane particles may be more subtle (Fig. 5). It is proposed that exposed peptides would have limited pathways for removal of heat and may become flattened and broadened. Such a hypothesis may account for the similarity of intramembrane particles from a variety of sources. For example, both enzymes reconstituted into the proteoliposomes in Fig. 5 appear globular, although independent studies show that the peptides incorporate as monomers (Costello et al., 1986) and that the enzymes differ significantly in molecular weight and peptide composition (cf. review by Kaback, 1985). For the preparation in Fig. 5 some effort was made to reduce heat input by shielding the surfaces from the warm-up of the electron gun and by depositing about 1.0 to 1.5 nm of metal in about 3 sec with a sample temperature of about -180°C. It is noteworthy that the two enzymes can be distinguished based on their diameters and on the fact that only the larger oxidase consistently produces complementary pits. We predict that pits corresponding to the smaller lac permease will become visible as the resolution and replica quality improves.

In addition to the heat deposited from the evaporation source, heat can be generated during the fracture and must be dissipated in the fracture surfaces. Recently, Gruijters and

Bullivant (1986) pointed out that heat released during fracture may be very high, resulting in a rise of surface temperature as much as 80°C. Heat from the fracture and evaporation steps are present in general and may be difficult to distinguish. However, the complementary replicas in Fig. 2 clearly demonstrate that heat can be deposited from the evaporation source because the surfaces, after fracturing is complete, are influenced differently.

Contamination from the vacuum chamber or from the evaporation source (Gross et al., 1978b) is an ever-present problem. The use of cryopumping in the vicinity of the sample (Steere, 1968; Bullivant and Ames, 1966) and ultra-high, oil-free vacuums (Gross et al., 1984; Escaig, 1984) and out-gassed guns (Gross et al., 1978a; Ruben, 1981) can greatly reduce the common sources of contamination. Material released during the fracture process and redeposited on the fracture faces cannot be controlled so easily. Such contamination can come from the ice or from trapped coolant which ideally should be pumped off above its melting point before the samples are fractured. Complementary replicas can help evaluate the extent of fracture contamination (see Fig. 3).

Complementary replicas have been invaluable in locating the plane of fracture through membranes. For example, the controversy over the location of the fracture plane through gap junctions was resolved with complementary replicas (Chalcroft and Bullivant, 1974). Likewise, replicas of Yamamoto et al. (1974) were very useful in confirming the earlier prediction by Clark and Branton (1968) that retinal rod outer segment disk membranes fracture within the hydrophobic bilayer cores. Simple lipid vesicles and proteoliposomes clearly demonstrate the bilayer fracture pattern of Branton (1966) by revealing the well-defined step from ice to the hydrophobic surface; complementary replicas provide additional support that the step is across the monolayer of lipid (see Fig. 6 in Fetter and Costello, 1986). Complementary replicas in Fig. 4 support the fracture model of the unusual fracture patterns seen in membranous cytochrome c oxidase vesicle crystals (Costello and Frey, 1982) in which hydrophilic surfaces of the 2-D crystalline arrays are exposed.

Acknowledgements

We are grateful to Drs. K. Matsushita and H.R. Kaback, Roche Institute of Molecular Biology, Nutley, N.J., for providing reconstituted proteoliposomes of lac permease and cytochrome o oxidase. We thank J. Graves for typing the manuscript. This work was supported in part by PHS Grant #GM27914 (to M.J.C.), #GM28750 (to T.G.F.) and a gift from R.J. Reynolds.

References

Abermann RM, Salpeter M, Bachmann L (1970) High Resolution Shadowing, in: Principles and Techniques of Electron Microscopy, Volume II, Hayat MA (ed.), Van Nostrand-Reinhold, Princeton, NJ, 197-217.

Aldrian AR, Horn HRF (1974) Trennung von Dampf und Warmostrahlung wahrend des auff ampfens beim thermischen bedampfen empfindlicher Objeckte, in: Proc. 8th Intl. Congr. Electron Microsc., Vol. 1, Australian Acad. Sci., Canberra, 406-407.

Belous MV, Wayman CM (1967) Temperature changes in thin metal films during vapor deposition. J. Appl. Physics 38, 5119-5124.

Branton D (1966) Fracture faces of frozen membranes. Proc. Nat. Acad. Sci. USA 55, 1048-1056.

Bullivant S, Ames A. (1966) A simple freeze-fracture replication method for electron microscopy. J. Cell Biol. 29, 435-447.

Chalcroft JP, Bullivant S (1974) An interpretation of liver cell membrane and junction structure based on observation of freeze-fracture replicas of both sides of the fracture. J. Cell Biol. 47, 49-60.

Clark AW, Branton D (1968) Fracture faces in frozen outer segments from the guinea pig retina. Z. Zellforsch. 91, 586-603.

Costello MJ (1980) Ultra-rapid freezing of thin biological samples. Scanning Electron Microsc. 1980; II: 361-370.

Costello MJ, Escaig J, Matsushita K, Carrasco N, Merrick D, Kaback HR (1986) Ultrastructure of frozen suspensions of proteoliposomes. Biophys. J. 49, 395a.

Costello MJ, Fetter RD (1986) Freeze-fracture methods: Preparation of complementary replicas for evaluating intracellular ice damage in ultrarapidly cooled specimens. Methods in Enzymol. 127, 704-718.

Costello MJ, Frey T (1982) Membranus cytochrome c oxidase: A freeze-fracture electron microscope analysis. J. Mol. Biol. 162, 131-156.

Costello MJ, Fetter RD, Corless JM (1984a) Optimum conditions for the plunge freezing of sandwiched samples, in: Science of Biological Specimen Preparation, Revel J-P, Barnard T, Haggis GH (eds.), SEM Inc, AMF O'Hare (Chicago), 105-115.

Costello MJ, Viitanen P, Carrasco N, Foster DL, Kaback HR (1984b) Morphology of proteoliposomes reconstituted with purified lac carrier protein from Escherichia coli. J. Biol. Chem. 259, 15579-15586.

Davy JG, Branton D (1970) Subliming ice surfaces: Freeze-etch electron microscopy. Science 168, 1216-1218.

Ellisman MH, Staehelin LA (1979) Electronically Interlocked Electron Gun Shutter For Preparing Improved Replicas of Freeze-Fracture Specimens, in: Freeze Fracture: Methods, Artifacts, and Interpretations, Rash JE and Hudson CS (eds.), Raven Press, New York, 123-125.

Escaig J (1984) Freeze-fracturing in ultrahigh vacuum: Low temperature sample holder (4.8K) and microtome sample holder UHV compatible, in: Proc. 42nd Electron Microsc. Soc. Am, Bailey GW (ed.), San Francisco Press, San Francisco, 2-5.

Fetter RD, Costello MJ (1986) A procedure for obtaining complementary replicas of ultra-rapidly frozen sandwiched samples. J. Microsc. 141, in press.

Frey T, Costello MJ, Karlsson B, Chan SHP, Hasselgrove JC, Leigh JS (1982) The structure of the cytochrome c oxidase dimer: Electron microscopy of two-dimensional crystals. J. Mol. Biol. 162, 113-130.

Gross H, Bas E, Moor H (1978a) Freeze-fracturing in ultrahigh vacuum at -196°C. J. Cell Biol. 76, 712-728.

Gross H, Kuebler O, Bas E, Moor H (1978b) Decoration of specific sites on freeze-fractured membranes. J. Cell Biol. 79, 646-656.

Gross H, Müller T, Wildhaber I, Winkler H, Moor H (1984) Freeze-fracturing and replication at -260°C, in: Proc. 42nd Electron Microsc. Soc. Am., Bailey GW (ed.), San Francisco Press, San Francisco, 12-15.

Gross H, Müller T, Wildhaber I, Winkler H (1985) High resolution metal replication, quantified by image analysis of periodic test specimens. Ultramicrosc. 16, 287-304.

Gruijters WTM, Bullivant S (1986) Freeze-fracturing at defined temperatures provides information on temperature rise during fracture, and on membrane complementarity. J. Microsc. (London) in press.

Gulik A, Aggerbeck LP, Dedieu JC, Gulik-Krzywicki T (1982) Freeze-fracture electron microscopic analysis of solutions of biological molecules. J. Microsc. 125, 207-213.

Kaback HR (1985) Proton electrochemical gradients and active transport: The saga of lac permease. Annals New York Acad. Sci. 456, 291-304.

Matsushita K, Patel L, Gennis RB, Kaback HR (1983) Reconstitution of active transport in proteoliposomes containing cytochrome o oxidase and lac carrier protein purified from Escherichia coli. Proc. Nat. Acad. Sci. USA 80, 4889-4893.

Matsushita K, Patel L, Kaback HR (1984) Cytochrome o type oxidase from Escherichia coli. Characteristics of the enzyme and mechanism of electrochemical proton gradient generation. Biochemistry 23, 4703-4713.

Muehlethaler K, Wehrli E, Moor H (1970) Double fracturing methods for freeze-etching, in: Proc. 7th Intl. Congr. Electron Microsc., Vol. 1, Société Francaise de Microscopie Électronique, Paris, 449-450.

Ruben GC (1981) The extent of platinum-carbon electron beam gun degassing before replication can change the size and frequency of replicated small particles. in: Proc. 39th Electron Microsc. Soc. Am., Bailey, GW (ed.), Claitor's Publ. Div., Baton Rouge, 566-567.

Steere RL (1968) Freeze-etching simplified, in: Proc. 26th Electron Micros. Soc. Am., Arceneaux CJ (ed.), Claitor's Publ. Div., Baton Rouge, 102-103.

Steere RL (1973) Preparation of high resolution freeze-etch, freeze-fracture, frozen surface, and freeze-dried replicas in a single freeze-etch module, and the use of stereo electron microscopy to obtain maximum information from them, in: Freeze-Etching Techniques and Applications, Benedetti EL, Favard P (eds.) Société Francaise de Microscopie Électronique, Paris, 223-255.

Steere RL, Moseley M (1969) New dimensions in freeze-etching, in: Proc. 27th Electron Microsc. Soc. Am., Arceneaux CJ (ed.), Claitor's Publ. Div., Baton Rouge, 202-203.

Watt IM (1974) Reduction in specimen level heating during carbon deposition by the Bradley technique. Proc. 8th Intl. Congr. Electron Microsc., Vol. 1, Australian Acad. Sci., Canberra, 402-403.

Yamamoto TY, Tonosaki A, Watanabe H (1974) Complementary faces of the rod disc membrane in freeze-fracture replicas. Tohoku J. exp. Med. 113, 313-317.

Zampighi G, Simon SA, Robertson JD, McIntosh TJ, Costello MJ (1982) On the structural organization of isolated bovine lens fiber junctions. J. Cell Biol. 93, 175-189.