Aclar discs: a versatile substrate for routine high-pressure freezing of mammalian cell monolayers

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Summary

High-pressure freezing avoids the artefacts induced by conventional chemical fixation, and, in combination with freeze-substitution and plastic embedding, is a reliable method for the ultrastructural analysis of mammalian cell monolayers. In order to high-pressure freeze mammalian cell monolayers, cells have to be seeded on a suitable substrate. Unfortunately, electron microscopy analysis is often hampered by poor cell growth, changes in cell morphology induced by the cell substrate or cell loss during processing. We report a method to culture, high-pressure freeze, freeze-substitute and plastic embed mammalian cell monolayers. The method is based on the use of Aclar, a copolymer film with properties very similar to those of tissue culture plastic. We show that Aclar discs support the normal growth and morphology of a wide variety of mammalian cell types, and form an ideal starting point for high-pressure freezing, freeze-substitution and plastic embedding. We present a complete protocol, which, because of its simplicity and reproducibility, provides a method suitable for the routine analysis of mammalian cell monolayers by electron microscopy and tomography.

Introduction

Mammalian cells in culture, frequently growing as monolayers, are specimens commonly used in cell biology. An important prerequisite for ultrastructural studies on these cell monolayers is the preservation of cellular fine structure in a state as close as possible to the native state. In this respect, cryofixation is the method of choice because it allows for a rapid immobilization of cellular components without altering their environment and thus avoids the artefacts induced by conventional chemical fixation (Vanharreveld et al., 1965; Gilkey & Staehelin, 1986). Among the cryoimmobilization methods, high-pressure freezing (HPF) is particularly powerful because it allows even relatively thick specimens to be frozen without the formation of large ice crystals (Müller & Moor, 1984). A widely used method combines high-pressure freezing with freeze-substitution (FS) to chemically fix and dehydrate the sample at a low temperature, followed by plastic embedding and thin sectioning (Humbel & Müller, 1986; Geerts et al., 2005).

In order to fix monolayers by HPF, cells first have to be grown on a substrate suitable for subsequent processing in a high-pressure freezer. In recent years, several cell substrates have been used in combination with cryofixation: gold electron microscopy (EM) grids coated with Formvar (Ladinsky et al., 1999), sapphire discs (Schwab, 1990; Eppenberger-Eberhardt et al., 1997; Hess et al., 2000), Thermax (Marsh et al., 2001), polyethylene terephthalate filter discs (Morpew & McIntosh, 2003), Mylar discs (McEwen et al., 1998) and HPF specimen holders (aluminium platelets) coated with Matrigel or polylysine (Sawaguchi et al., 2002, 2003). Although, in principle, each of these substrates can be used to grow and freeze cell monolayers by HPF, success is often limited due to the practical difficulties encountered during the handling and processing of samples. In addition, most of these substrates have properties quite different from ordinary tissue culture plastic, they generally do not support growth of all cell types and potentially induce changes in morphology as compared to that of cells grown on tissue culture plastic.

The aim of the current study was to develop a routine method for the ultrastructural analysis of mammalian cell monolayers that can be applied to a wide range of cell types. As our starting point we chose Aclar® 33C (Honeywell, Morristown, NJ), a plastic film made from fluorinated-chlorinated resins with many properties in common with ordinary polystyrene.
tissue culture plastic. Aclar has been successfully used to culture several mammalian cell types, and is nontoxic even after long-term culture (Kingsley & Cole, 1988; Masurovsky & Bunge, 1989; Meyer et al., 1998). Aclar film can be punched to the desired shape and size, and is easy to handle because it does not break during manipulation and remains flat. Importantly, Aclar is inert to chemicals commonly used in specimen preparation, such as acetone, methanol and osmium tetroxide, and can thus be used for EM applications (Masurovsky & Bunge, 1968; Kingsley & Cole, 1988). Because Aclar is crystal clear, cells can be monitored during culture. Moreover, it can be easily peeled off from polymerized Epoxy resin leaving a perfectly smooth surface; alternatively, it can also be sectioned together with the sample without damaging the ultramicrotome knife (Kingsley & Cole, 1988). Not surprisingly, Aclar has been widely used for transmission and scanning EM studies in combination with chemical fixation (Kingsley & Cole, 1988; Masurovsky & Bunge, 1989). However, because it does not become brittle but remains flexible even at liquid nitrogen temperatures it can also be used in cryofixation procedures (Burry & Lasher, 1978; Schwarb, 1990; Cook, 2004).

Encouraged by the promising features of Aclar, we developed a protocol to culture, high-pressure freeze and freeze-substitute mammalian cell monolayers. Sample handling, cryofixation and FS procedures were optimized to reduce cell loss, increase freezing quality and ultrastructural preservation, and allow easy identification and serial sectioning of the cells of interest. The method is simple and highly reproducible. Because it can be applied to a wide range of mammalian cell types, our method should prove an excellent starting point for routine ultrastructural analysis of mammalian cell monolayers.

Materials and methods

Preparation of culture substrates

Films of 2 mil (51 µm) thick Aclar®33C were purchased from Electron Microscopy Sciences (#50426-10; EMS, Fort Washington, PA). Substrates for cell culture were prepared according to the following protocol. Wearing gloves:

• A ~5 × 5 cm piece of Aclar film was cut (with ordinary scissors) and both sides were cleaned using a soft paper tissue moistened with acetone;
• 3 mm discs were punched out using a household revolving punch plier just pressing to make incisions but without separating the discs from the Aclar sheet (Fig. 1A);
• The discs were pulled out one by one from the sheet, taking care that the forceps only touched the edges of the discs (Fig. 1B); discs were stored in glass containers;
• Using forceps, the Aclar discs were placed in tissue culture dishes (eight discs in a 35 mm dish). To prevent flipping and movement of the discs and facilitate next sample handling, the discs were attached to the dish using a hot metal wire fastened to a wooden stick (Fig. 1C). Each disc was first pressed onto the tissue culture dish using a micropipette tip (Fig. 1C), and then touched at three points (Fig. 1D, insert) along its edge with the hot metal wire;
• The location of the discs was marked on the outside of the culture dish (Fig. 1D), and the dishes were sterilized in a microwave oven (5 min, 800 W); the dishes were sealed with Parafilm (Pechiney Plastic Packaging, Chicago, IL) and stored.

Using microwave sterilization as indicated, we never experienced any contamination during culturing:

• Just before seeding cells, the discs were incubated in supplement-free culture medium for 2 h at 37 °C to wash them and improve their hydrophilic character. If required (for human umbilical vein endothelial cells (HUVECs), see below) the discs were precoated with fibronectin following standard procedures.

Cell culture

Epithelial cell lines (NRK52E, HeLa, CHO and Caco-2), fibroblast cell lines (COS-7, NIH-3T3 2.2 and HER-14), primary cultures of human skin fibroblasts (here called HFs) and primary cultures of HUVECs were grown in tissue culture flasks following standard procedures (5% CO₂, 37 °C). HUVECs were cultured in EGM-2 (# CC-3162; Cambrex, Walkersville, MD) after precoating the tissue culture plastic with fibronectin. The other cell lines were cultured in Dulbecco’s Modified Eagle Medium (DMEM) (Invitrogen, Paisley, UK) supplemented with 10% Fetal Bovine Serum (FBS) (10% calf serum for HF), l-glutamine, penicillín-streptomycin and, in the case of NRK52E, also including sodium pyruvate (Invitrogen).

Cells were harvested by trypsinization from flasks containing 80% confluent cultures, and seeded in dishes containing the Aclar discs after first removing the supplement-free culture medium used in the preincubation of the Aclar discs. Dilutions were chosen to obtain 70–90% confluent cultures 2 days after seeding. Special care was taken to avoid moving the dishes during the first few hours of incubation. The next day, the medium was refreshed and cells were further incubated for 24 h. Cell growth and morphology were monitored by phase contrast light microscopy, and only Aclar discs with a cell confluence of 70–90% and without apparent anomalies were used for experiments. In a typical experiment to study the interface between the endosomal system and the Golgi complex, NRK52E cells grown on Aclar discs were washed with DMEM-HEPES (DMEM buffered with 25 mM HEPES, pH 7.4) at 37 °C and transported to the HPF room. Cells were incubated with Bovine Serum Albumin (BSA) conjugated to 5 nm gold particles in DMEM-HEPES (containing 0.5% FBS) for 10 min at 37 °C in a water bath to label the endocytic compartments, and subsequently high-pressure frozen.

High-pressure freezing

For HPF, Aclar discs were sandwiched between Ø 3 × 0.5 mm aluminium platelets (M. Wohlwend, Sennwald, Switzerland).
A platelet with two cavities, Ø 2 × 0.2 mm and Ø 2 × 0.1 mm, was mounted in the HPF specimen holder with the shallow cavity upwards (lower platelet in Fig. 1E). This cavity was filled with a nonpenetrating cryoprotectant, 20% (w/v) dextran (MW, ∼40 kDa; #31389, Fluka, Buchs, Switzerland) (Al-Amoudi et al., 2002). Before use, this dextran solution in culture medium (without supplements) was dialysed overnight against culture medium to remove any low molecular weight contaminants. A dish with Aclar discs was taken from the 37 °C water bath. A light lateral pressure was applied on the edge of an Aclar disc to separate it from the dish using forceps. The disc was then dipped into cryoprotectant at 37 °C and immediately transferred to the specimen holder, with the cells facing down (Fig. 1E). A binocular microscope was used.

Fig. 1. (A–B) Preparation of Aclar discs. (C) Details of the tools used to arrange and attach Aclar discs. The use of a plastic tip to manipulate the Aclar discs avoided damaging their surface. (D) A 35 mm dish with eight Aclar discs attached with their location marked on the outside of the culture dish. The insert shows a detail of an Aclar disc with its three points of attachment. (E) Schematic drawing of the sample sandwich used for high-pressure freezing (HPF). The Aclar disc with cells facing down was put on top of the lower platelet; the cavity of this platelet was filled with a 20% dextran solution. (F) Self-made basket [for housing sandwiches during freeze-substitution (FS)] containing crushed aluminium rings (arrow). Insert: detail of aluminium rings before (up) and after (down) being crushed. For details see text.
to ensure that the sample did not contain any air bubbles that might interfere with proper freezing. A second platelet with a single cavity (Ø 2 × 0.3 mm) was placed on top of the Aclar with its flat surface facing down (Fig. 1F). The specimen holder was closed and the samples were frozen from a temperature of 30 °C using a Leica high-pressure freezer (Leica EM HPF; Leica Microsystems, Vienna, Austria; now M. Wohlwend, Sennwald, Switzerland) at a pressure of about 2000 bar. Samples were high-pressure frozen within 30 s from the time of removal of the Aclar disc from the culture dish. After freezing, the intact ‘sandwich’ was stored in liquid nitrogen.

Freeze-substitution and embedding

In order to prevent cell loss during the procedure, a housing system was designed that protects the samples while allowing sufficient fluid exchange. To this end, FS was carried out in microtubes (1.5 mL; #72.690, Sarstedt, Nümbrecht, Germany) containing self-made baskets. These baskets were prepared by removing the top and bottom part of a 0.5 mL microtube (#72.699, Sarstedt) using a razor blade, leaving a hole of 0.5–1 mm at the bottom. Two crushed aluminium rings (Ø 3 × 2 × 0.2 mm; #X0026621, J eveka bv, Amsterdam, the Netherlands) (Fig. 1F, insert) were inserted per basket (Fig. 1F). FS fluid containing 1% osmium tetroxide (EMS), 0.5% anhydrous glutaraldehyde (EMS) and 1% H₂O₂ (Walther & Ziegler, 2002) in anhydrous acetone (#1.00299.0500; Merck, Darmstadt, Germany) was freshly prepared, distributed over the microtubes (1 mL per tube) and immediately frozen in liquid nitrogen. Intact high-pressure frozen sandwiches were transferred under liquid nitrogen to the microtubes (four per tube) and placed in a FS machine (CS auto; Reichert-Jung; now Leica Microsystems, Vienna, Austria) precooled to −90 °C. After 1 h, the FS fluid had melted and the sandwiches had sunk to the bottom of the basket on top of the aluminium rings. There, the sandwiches slightly opened up but did not disassemble. Freeze-substitution was carried out at −90 °C for 48 h. Subsequently, the samples were warmed up (Studer et al., 2001), kept on ice for 1 h (Wild et al., 2001) and washed in anhydrous acetone on ice (30 min, four times). Next, cells were infiltrated with Epoxy resin (#45359; Fluka). Baskets were incubated at 4 °C in microtubes with acetone : Epoxy resin mixtures (2 : 1 for 4 h; 1 : 1 overnight; 1 : 2 for 4 h; 1 : 3 for 4 h), transferred to Epoxy resin, and left overnight at room temperature. In each step from washing in acetone to resin infiltration, the baskets were transferred to new 1.5 mL microtubes containing the fluids where they were first gently immersed and moved up and down several times. The rings present in the baskets prevented the sandwiches from blocking the (hole at the) bottom of the basket. This guaranteed free fluid exchange between the basket and microtubes while at the same time minimizing the loss of cells.

Finally, sandwiches were taken out of the baskets and disassembled in a dish with Epoxy resin. Because the sandwiches maintained their original platelet–Aclar–platelet arrangement, the location of the cell monolayer was known. Aclar discs with cells facing upwards were transferred to a new dish with a 2-mm-thick layer of fresh Epoxy resin for flat embedding. A 1-cm-long plastic cylinder, prepared from a Beem embedding capsule (#69911; EMS; bottom and lid cut off using a razor blade), was immersed in the resin on top of every Aclar disc. After resin polymerization (overnight at 60 °C) the cylinders were filled up with fresh Epoxy resin and left at 60 °C for another 24 h. Finally, the polymerized blocks, with Aclar discs on their surface, were separated from the dish.

Localization of cells, sectioning and poststaining

The plastic blocks were trimmed to completely expose the Aclar discs, which could then be easily separated from the polymerized Epoxy resin, leaving behind a perfectly smooth surface containing the plastic-embedded cells. With the help of a binocular microscope and a potent light source, an area containing lightly brown stained cells was localized, and after proper trimming, the cell monolayers were sectioned either obliquely or en face. Thin (60 nm) sections were collected on 50-mesh Formvar-carbon-coated copper grids for routine EM investigation. For electron tomography, 250-nm-thick serial sections were transferred to Formvar-carbon-coated copper slot grids. Sections were stained in a saturated solution of uranyl acetate in 70% methanol followed by Reynolds’ lead citrate. For electron tomography, colloidal gold particles (15 nm) were applied to one side of the grid to serve as fiducial markers in the alignment of the tilt series.

Electron microscopy and electron tomography

Thin sections were examined on a Tecnai-12 (FEI Co., Eindhoven, the Netherlands) operating at 100 kV equipped with a side-mounted Megaview II camera (SIS, Münster, Germany). For electron tomography, tilt series were automatically recorded (Ziese et al., 2002) at 200 kV using a Tecnai-20 equipped with a bottom-mounted slow-scan CCD camera (Tem-Cam F214; TVIPS GmbH, Gauting, Germany) and a motorized goniometer. Specimens were tilted about two orthogonal axes at 1° intervals from −65° to +65°, resulting in two datasets of 131 high-resolution digital images (1 × 1 K). Images were aligned, tomograms computed and the two single-axes tomograms merged into one using the program package IMOD (Kremer et al., 1996; Mastronarde, 1997).

Results and discussion

A method suitable for routine ultrastructural analysis of mammalian cell monolayers processed by HPF and FS should meet the following criteria: (1) the cell substrate allows for normal growth and morphology of a large number of mammalian cell types; (2) HPF results in excellent structural preservation.
with large ice crystals being absent in the majority of cells; (3) FS allows for proper dehydration and plastic embedding whilst also preserving cellular morphology and offering sufficient (membrane) contrast; (4) the method is simple and practical, and has a high success rate with minimal loss of cells. In this technical note we describe a complete procedure that meets all of these criteria. This method is based on the use of Aclar as cell substrate.

**Aclar provides a versatile substrate for cell culture**

We tested our method most extensively on normal rat kidney cells (NRK52E). Among the other widely used mammalian cell lines that we tested, all (i.e. HeLa, CHO, Caco-2, COS-7, NIH-3T3 2.2, HER-14 and primary HF) except primary HUVECs adhered well to the Aclar discs. Representative examples, indicating identical morphology and growth of cells on Aclar discs as compared to the surrounding surface of the tissue culture dish, are shown in Fig. 2. Because Aclar provides an excellent substrate for cell growth of many different mammalian cell lines (also see Kingsley & Cole, 1988; Masurovsky & Bunge, 1989; Meyer et al., 1998), with properties quite comparable to those of tissue culture plastic, Aclar discs are an ideal starting point for the EM analysis of mammalian cell monolayers.

**HPF of cells grown on Aclar discs resulted in excellent structural preservation with large ice crystals being absent in the majority of cells**

Cells had well-preserved nuclei and cytoplasmic organelles (Fig. 3A–D: thin sections, Fig. 3E: tomographic slice; NRK52E Fig. 2. Phase contrast light microscopy of cells grown on Aclar and tissue culture plastic. Morphological differences were not observed between cells attached to Aclar (on the right-hand side of each panel) as compared to cells attached to the tissue culture dish (on the left-hand side). Pictures were taken from neighbouring areas in the Aclar disc and tissue culture dish.
Fig. 3. Ultrastructure of high-pressure frozen, freeze-substituted and Epoxy resin-embedded NRK52E cells. (A–D) Thin-section EM of cells, sectioned obliquely (A, boxed area detailed in B) or en face (C, boxed area detailed in D) with respect to the block surface. The cells have well-preserved organelles and cytoskeleton: euchromatic nuclei showing segments of the nucleolus (n) and nuclear pores (black arrows), mitochondria (m), endoplasmic reticulum (er), Golgi profiles (g), clathrin-coated vesicular profiles (white arrows in B), elements of the endosomal–lysosomal system (e, l), microtubules (black arrowheads) and actin filament bundles (white arrowheads in B). Note the endosomes containing endocytosed gold (e'). In (A), the dextran (white asterisk) and the original location of the Aclar before it was peeled off (black asterisk) are indicated. (E) Tomographic slice (2 nm thick) extracted from a dual-axes 3D reconstruction of a Golgi–endosome interface. An early endosome is easily identified by the presence of gold particles in its lumen (e'). Membranes, microtubules (black arrowheads) and actin filaments (white arrowheads) are well resolved. Scale bars: A and C, 2 μm; B and E, 0.5 μm; D, 1 μm.
cells). Many ultrastructural fine details such as clathrin-coated buds and vesicular profiles as well as cytoskeletal elements (microtubules and actin filaments) were clearly resolved (Fig. 3B). In our standard protocol, dextran was used as a nonpenetrating cryoprotectant to avoid extracellular ice crystal formation (Al-Amoudi et al., 2002). A minor drawback was the formation of a dextran-rich shell surrounding the cells, which may result in cutting artefacts at the boundary between the cells and the dextran, occasionally obscuring ultrastructural details near the plasma membrane (data not shown). However, using this procedure greatly improved the yield of properly frozen cells and cellular ultrastructure was well preserved in the majority of cells. Thus, cell monolayers on Aclar discs can be properly high-pressure frozen.

**FS and low-temperature chemical fixation of cell monolayers on Aclar discs preserves cellular morphology and offers high membrane contrast**

In initial experiments FS was carried out using fixatives in anhydrous acetone, and cellular membranes were poorly visualized despite the fact that cells were properly frozen (data not shown). In subsequent experiments, water was added to the FS medium (Walther & Ziegler, 2002). Adding 1% water greatly improved contrast and membranes were clearly resolved (Fig. 3). However, in the presence of water in the FS medium, the dextran, because of its hygroscopic properties, formed a hydrated shell around the cells hindering dehydration and subsequent resin impregnation. Fortunately, this problem could be minimized by washing intensively with acetone and mixtures of acetone and Epoxy resin before fixation and subsequent resin impregnation. Fortunately, this problem could be minimized by washing intensively with acetone and mixtures of acetone and Epoxy resin before plastic embedding, always taking special precautions to limit cell loss (see Materials and methods).

**The Aclar method is simple and practical, and has a high success rate with minimal loss of cells**

Aclar discs are easy to make (over 200 discs can be prepared in 1 h) and can be stored indefinitely. Because Aclar is a plastic, static electricity may arise. However, this problem can be overcome by wearing an antistatic bracelet on the wrist during disc preparation. Aclar has many useful characteristics. (1) The discs can be attached to the tissue culture dish by applying local heat, i.e. without using adhesives that might interfere with normal cell growth. After attachment, the Aclar discs cannot move, facilitating seeding of cells, media refreshment, transportation of the dish and preparation of HPF sandwiches. (2) Because Aclar is crystal clear, cells can be monitored during culture, and the best discs can be selected for HPF. (3) The thickness of the Aclar used in the experiments (51 µm) results in flexible discs thick enough to remain flat. A flat surface is essential for optimal cell attachment for many cell types, whereas the flexibility of the substrate facilitates its handling by preventing breakage or permanent deformation. (4) Aclar peels off from polymerized Epoxy resin leaving a perfectly smooth surface where cells can be observed using a binocular microscope and a proper light source. Despite optimization of the HPF procedure, not all cells were properly frozen. However, with some practice, groups of well-frozen cells could be easily identified in the face of the plastic block. Whereas darkly stained (black) groups of cells showed extensive ice crystal damage (data not shown), lighter (brown) stained cells were usually well preserved (Fig. 3). Importantly, the smooth surface of the block left after Aclar peeling allows for an easy en face serial sectioning of complete cells, which is crucial in electron tomography of the secretory pathway. (5) Aclar can be easily marked (Masurovsky & Bunge, 1971) and does not exhibit any autofluorescence (Kingsley & Cole, 1988) turning it into an ideal substrate for correlative light-electron microscopy.

The protocol we have described in the current paper is now being routinely used to analyse the interface between the Golgi and endocytic systems in NRK52E cells. We typically find that at least six out of eight Aclar discs (per dish) contain cells at the desired 70–90% confluence, as long as movement of the dish with Aclar discs in the first few hours after cell seeding is avoided. In addition, it is important to preincubate the Aclar discs with nonsupplemented medium, which apparently improves the hydrophilic character of the Aclar surface, facilitating cell attachment and growth. From every dish, the best Aclar discs are selected and high-pressure frozen, taking special care that the sample sandwiches do not contain any air bubbles. It is important to keep the sample sandwiches ‘intact’ during FS and subsequent washings to protect the samples and to prevent Aclar discs from floating. During freeze-substitution the sample sandwiches are not touched directly but housed in self-made baskets. Because the sandwiches slightly open up during FS, and the baskets allow for a sufficient but gentle fluid exchange (see Materials and methods) cells can be properly freeze-substituted and resin-infiltrated whilst keeping cell loss to a minimum. The success rate of our protocol is high, with over 90% of the plastic blocks containing areas with well-frozen and properly contrasted cells. Furthermore, blocks can be easily split and re-embedded, allowing EM analysis of as many cells as possible.

In conclusion, Aclar provides an excellent substrate for a wide variety of mammalian cell types, and forms an ideal starting point for high-pressure freezing and freeze-substitution. The procedures presented in this paper provide a method for the routine analysis of mammalian cell monolayers by electron microscopy and tomography.

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