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Short Technical Note

A simplified 'sandwich' technique for *in situ* embedding and perpendicular sectioning of monolayer cultures of human skin fibroblasts

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SUMMARY

In the processing of cell cultures, grown as a monolayer in tissue culture dishes for electron microscopy, the sectioning of the monolayer is an essential step. The monolayer can be sectioned either parallel or perpendicular to the plane of growth. Several methods for the perpendicular way of sectioning have already been described. We propose a simplified method in which the monolayer is sandwiched between two layers of resin, one of which is a prepolymerized block, the other being a layer of resin, applied at a second stage. Sectioning of this 'flat embedded' specimen yields thin sections perpendicular to the plane of growth of the monolayer without elaborate orientating procedures. The advantage of this procedure is that it can be done using only routine embedding techniques, avoiding special materials or complex manipulations. This sandwich technique provides an excellent mechanical fixation of the monolayer and protects it against external damage.

INTRODUCTION

In studying monolayer cell cultures by electron microscopy, *in situ* embedding and sectioning of the cells provides the opportunity to keep them in their substrate-linked situation and to visualize them in their intercellular context. One can section the monolayer either parallel or perpendicular to the substrate, both ways of sectioning being complementary in one's endeavours to reconstruct the three-dimensional morphology of the cells. Perpendicular sections have the advantage of giving an impression of the third spatial dimension of the cells, and of visualizing in a very crisp way the intercellular relationships as well as the cellular membrane.

Before plastic Petri dishes were readily available, cells were grown on glass surfaces. The cell cultures tended to stick very strongly to these surfaces, making it very difficult to separate them from each other (Gay, 1955; Borysko, 1956). Therefore, a variety of procedures have been developed to solve this problem: glass surfaces have been coated with special materials prior to culturing to facilitate detachment; materials other than glass were used that could be separated more easily from the embedding resins; methods were proposed which used materials that could be sectioned together with the cell monolayer, so that detachment was no longer needed and the whole (substrate+monolayer) could be sectioned perpendicular to the substrate. Many of these techniques are summarized by Nelson & Flaxman (1972). A comprehen-

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sive survey of methods for the embedding and sectioning of monolayers was made by Glauert (1974).

Recent technological developments have made available synthetic materials such as Melinex polyester film (Agar Scientific, Stansted, Essex), Thermanox tissue culture coverslips (LUX, Miles Lab., Naperville, Ill.) and Permanox tissue culture dishes (LUX, Miles Lab.), which facilitate easy separation from polymerized resin (Azim *et al.*, 1981; Langanger *et al.*, 1984; Bowen Jones & Gray, 1985; Townes-Anderson *et al.*, 1985). These materials have the additional advantages that cells can be grown directly on them by standard methods of culture and they are resistant to all commonly used solvents. Taking advantage of these characteristics by using Permanox dishes to culture human skin fibroblasts, we propose a simplified method for *in situ* embedding and perpendicular sectioning of cell monolayers.

PROCEDURE

Human skin fibroblasts were grown on LUX CONTUR Permanox tissue culture dishes (Miles Lab.), using Eagles' MEM medium supplemented with 10% fetal calf serum, 1% glutamin, 1% antibiotics (Peni/Strepto) and buffered with HEPES, pH 7·4 (all GIBCO Ltd, Paisley, U.K.). At confluence, the cells were processed for electron microscopy. The growth medium was removed by two washes with phosphate-buffered saline (PBS), pH 7·4, and subsequently replaced by 3% glutaraldehyde fixative, buffered with phosphate buffer to pH 7·4. After 1 h of fixation at room temperature, the dishes were rinsed with PBS for 30 min. Post-fixation was carried out with aqueous osmium tetroxide (1%) in phosphate buffer (pH 7·4) for

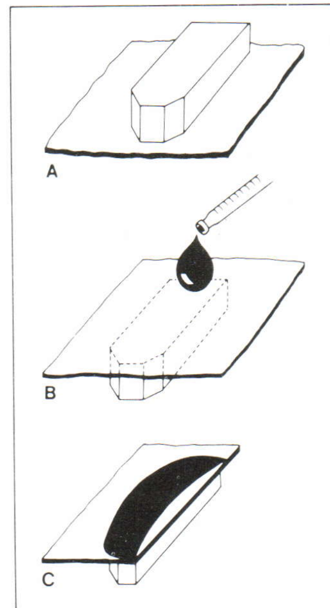

Fig. 1. Consecutive steps in the sandwich embedding technique (for the purpose of clarity, proportions are not depicted in a realistic way!). (A) After placing prepolymerized blocks of resin on the resin-impregnated monolayer in the culture dish, the whole is cured, the sheet of resin is removed from the dish and broken at a distance around each prepolymerized block, leaving sheet-fragments in which the cells are embedded and on top of which a prepolymerized block is fixed. (B) A drop of resin is spread on the cell-bearing side and the whole is cured. (C) To demonstrate the final result, a longitudinal section through the specimen is represented showing that the monolayer is sandwiched between two layers of hardened resin (the meniscus being exaggerated for clear demonstration).

1 h. After another wash with PBS, the specimen was dehydrated in increasing concentrations (70–90–100–100%) of ethanol. Propylene oxide was used as transitional solvent before filling the entire culture dish with epoxy resin (LX-112, Cat. 21210; Ladd Research Industries, Burlington, Vermont). The cultures were kept overnight in this condition in order to let the resin penetrate into the monolayer. After one night the superfluous resin was removed. Prepolymerized blocks of resin (prepared in moulds for flat embedding: 'Flat Silicon Rubber Mould'; Ladd Research Industries) were then placed on the resin-impregnated monolayer and

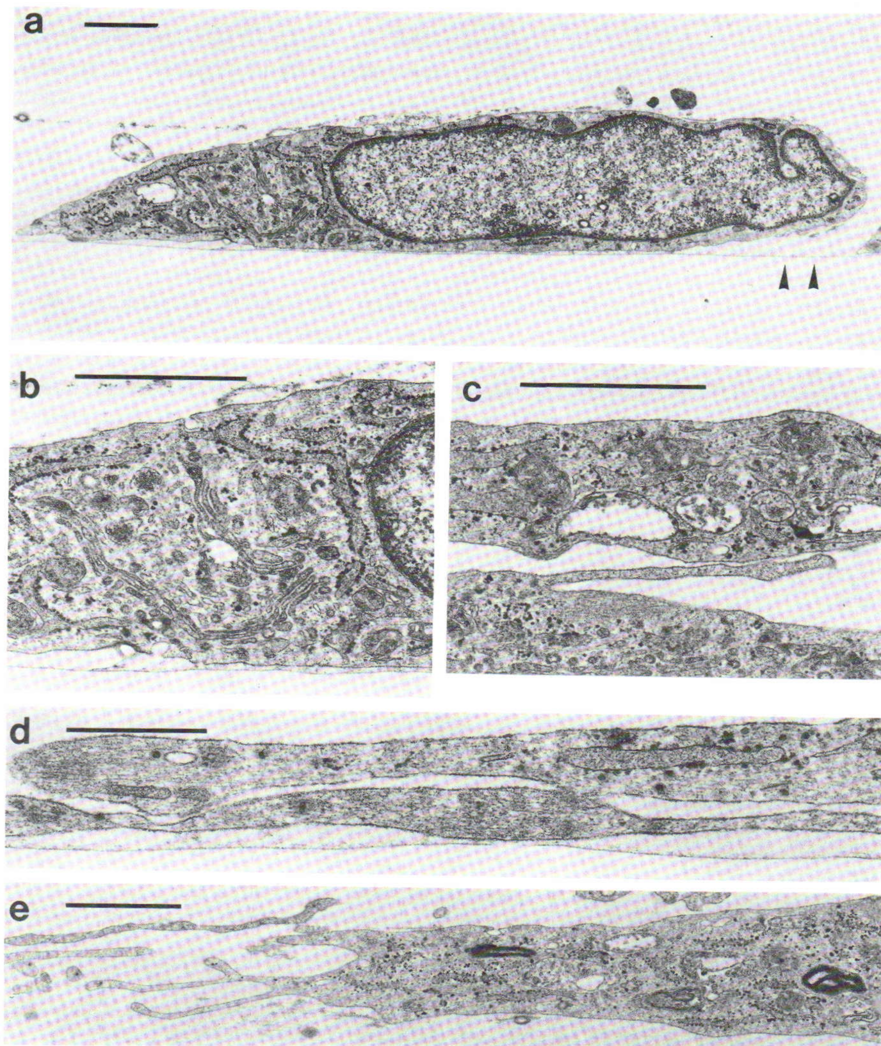

Fig. 2. Electron micrographs of monolayers of human skin fibroblasts, sectioned perpendicular to the substrate. (a) A fibroblast at low magnification. The line of demarcation (arrow heads) between the cell-bearing side of the impregnated specimen (top) and the drop of resin, applied at a second stage (bottom) is visible. (b) Detail of (a), showing part of the nucleus, the Golgi region, intracellular filaments, RER and plasma-membrane invaginations. (c), (d) Demonstration of the intercellular relationships which are not as clear in sections parallel to the substrate. (e) A micrograph showing the ruffling of the cell margin, where the fibroblast projects multiple extensions at its periphery. This view of the third dimension of the cell adds interesting information to the two-dimensional view yielded by parallel sections alone. Bars in (a), (b), (c) and (e): 1 μm; bar in (d): 0·5 μm.

the whole was cured at 333 K for 60 h. The hardened resin could be easily removed from the Permanox culture dish, resulting in a thin sheet of resin in which the cells were embedded and on top of which the prepolymerized blocks were fixed. The sheet was broken around each prepolymerized block. The edges were cut away, leaving blocks of resin, geometrically defined by the prepolymerized blocks, and consisting of the blocks, fixed on the sheet-fragments in which the cells were embedded.

Sections (1 μm thick) were cut from these blocks. After Toluidine Blue staining, light-microscopical observation did not reveal the cells clearly. In electron micrographs of thin sections of these specimens, the cells appeared to be hidden in the curls of the section. Apparently the very superficial position of the cells in the thin section led to mechanical damage during handling of the blocks when trimming and sectioning. It was therefore essential to protect this delicate surface immediately after removing it from the Permanox dish. To achieve this goal we changed our technique into a 'sandwich' technique for perpendicular sectioning. After breaking the sheet of resin at a distance around each prepolymerized block, the sheet was wetted with propylene oxide on the cell-bearing side, and a drop of resin was placed on it, so that the monolayer was sandwiched between two resin layers (Fig. 1). After curing, trimming, sectioning and staining, the monolayer was well preserved and could be studied in thin sections in the electron microscope (Fig. 2). The sandwiching of the monolayer prevents curling of the ultrathin sections and provides good protection against any external damage to the cells.

The great advantage of this technique is the simplicity with which perpendicular sectioning of cultured monolayers can be realized. Cells can be cultured in the Permanox dishes without using coverslips or other special materials. As a consequence of the flat surfaces of both the culture support and the prepolymerized blocks, there are no orientation problems and perpendicular sections through the monolayers are readily obtained. The number of manipulations is reduced to a minimum, and they do not differ from the routine embedding manipulations in electron microscopy.

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