Kawasaki Med. J. Vol. 6: No. 3·4, 93-100, 1980

# A SIMPLE TECHNIQUE FOR OBSERVATION OF SURFACE MORPHOLOGY OF VARIOUS CELLS CULTIVATED *IN VITRO*: THE SURFACE REPLICA TECHNIQUE

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*Accepted for Publication on June, 21, 1980*

## Abstract

**A simple technique using critical point drying and ordinary shadowcasting techniques was established for the replication of complicated cell surfaces into metal membranes. The results indicate that this technique is available for examination of the cell surface morphology at higher resolution than previously possible and that the stereographic analysis of cell surface morphology is available by use of this technique.**

## INTRODUCTION

Scanning electron microscopy has been widely applied in the examination of the surface morphology of animal cells and tissues, presumably due to its focus depth and facility in the specimen preparation. However, it suffers from a limited resolution imposed by the resolution of the microscope itself and the thickness of carbon and metal coating necessary to avoid the electric "charge-up" in the specimen. To examine not only cell surface morphology, but also biological activities using fine markers with specific activities under the transmission electron microscope, replica technique capable to covering wide area of complicated cell surface has been desired. A simple technique using critical point drying and shadowcasting techniques is reported in this paper.

## MATERIALS AND METHODS

Cells used in this experiment were L, HeLa, Vero, WI-38 cells[1] and fibroblasts at 15th pdl (population doubling level) isolated from adult human skin tissue (M. Namba, Dept. Exp. Pathol., Kawasaki Med. Sch.). All cells were cultivated *in vitro* on coverslips (5×5 mm). Replication procedure is shown in Fig. 1. The cells grown were washed twice in warm phosphate-buffered saline, pH 7.4 [PBS(+) of Dulbecco and Vogt[2] containing Ca and Mg] and

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dipped in Karnovsky's aldehyde fixative[3] or 2.5% glutaraldehyde in PBS(−) [phosphate-buffered saline but lacking Ca and Mg] for 15-20 min at room temperature. Results obtained in preliminary experiments showed that the cell surface fixed only with glutaraldehyde or Karnovsky's fixative was very rough and many cracks were occurred in the cells. After 2 time-washing in PBS(−), the cells were refixed in 1% osmium tetroxide in PBS(−) for 30 min at room temperature, dehydrated in an ethanol series [25, 40, 50, 70, 90, 95, and 100%, for 10 min each] and then dried in a Hitachi HCP-1 type critical point drying apparatus using $CO_2$ after dipping in *iso*-amyl acetate for 5 min. The coverslip was set at an angle of 30° against evaporating source [commercial 7 time-twisted basket of 0.5 mm$\phi$ tungsten filament] on the rotary stage in a Hitachi HUS-4GB vacuum evaporator, shadowcast with platinum-palladium alloy [2 cm of 0.1 mm$\phi$ filament] at a distance 8 cm from the specimen, then carbon [commercial rod 5 mm$\phi$ sharpened into 1 mm$\phi$, 6mm in length] was evaporated at 10 cm from the specimen rotating at 60 rpm. The coverslip was dipped in distilled water, then commercial bleaching solution containing sodium hypochlorite or 40% NaOH solution was added to about 20% for washing out the cell component. The replica membrane was kept overnight in the washing solution and rinsed several times in distilled water, collected on a copper grid and examined with a Hitachi H-500 electron microscope at the accelerating voltage of 75 KV. The replica membrane in the same size as that of the coverslip was readily obtained when carbon was evaporated up to about 200Å in thickness.

For three dimensional observations of cell surface morphology, the stereopairs

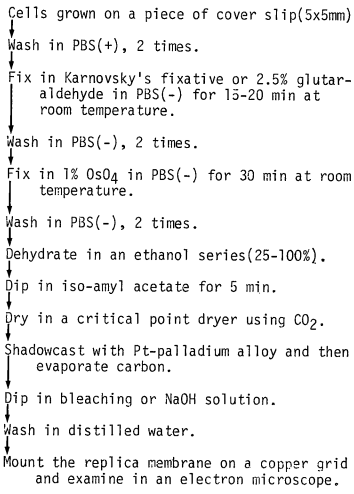



Fig. 1. Procedure of surface replica technique.

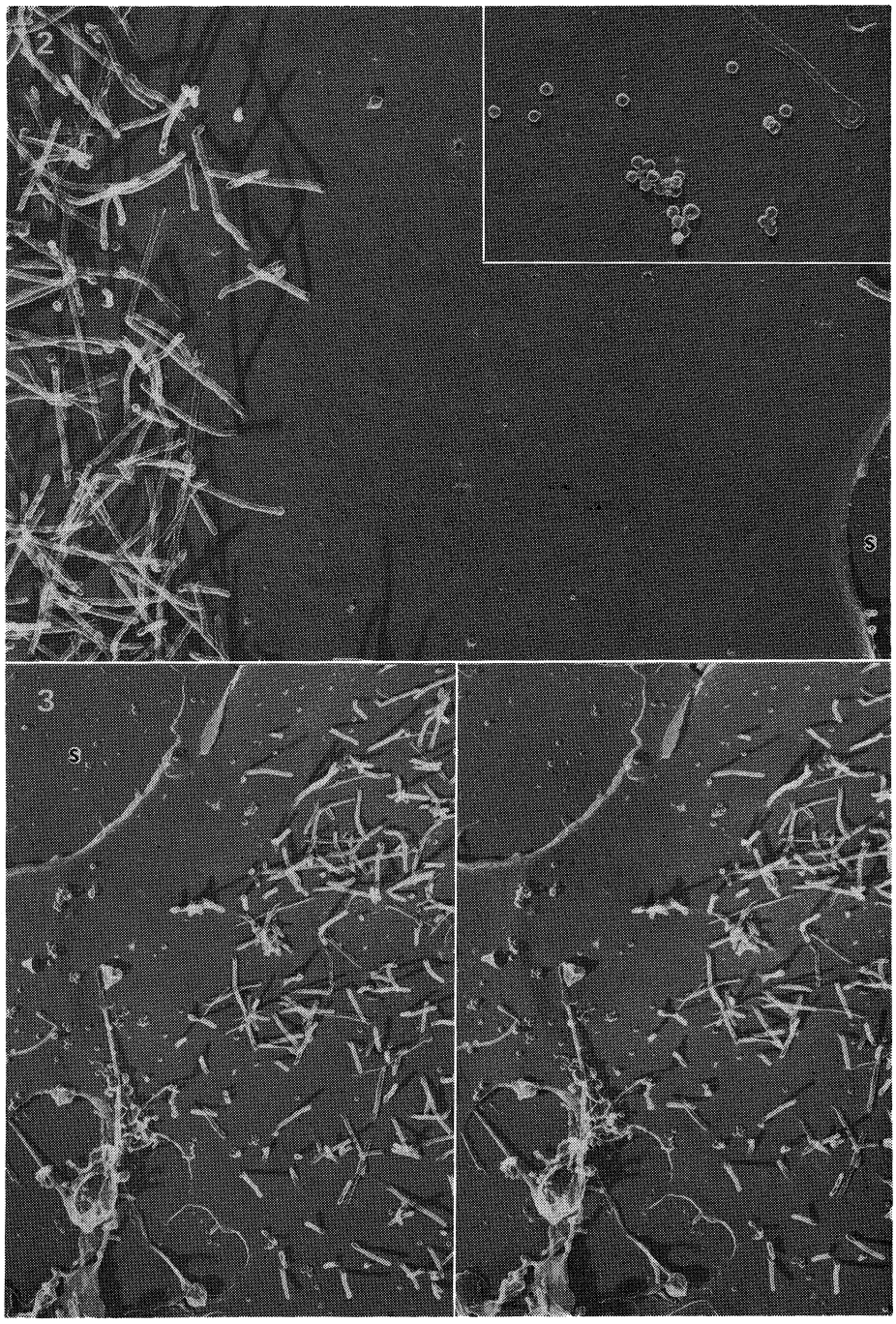
of micrographs were taken with a Hitachi HU-12A electron microscope at tilting angle of $\pm 10°$ and viewed stereographycally.

## RESULTS

Fig. 2 is a part of L cell which is characterized by a number of microvilli and a very smooth, flat surface. Many spheres, about 120 nm in diameter, were frequently observed on the smooth surface (inset). These may be C type virions produced spontaneously. When the cell was viewed stereographycally, it was clearly shown that the virions scattered at random on the surface. Some of them formed small clusters and necklace-like chains, suggesting successive budding at the "hot spots"[5] of virion assembly (Fig. 3). Microvilli, thinly elongated or globular projections were observed three-dimensionally as the thin membrane with clear shadows. In contrast to L cells, the morphological differentiation of the surface was not clearly seen in HeLa cells. Their whole surface was covered with a tremendous number of microvilli, which lacked uniformity in length and showed a larger diameter than those in L cells (Fig. 4). It was noted that neighboring cells touched each other with a number of finger-like projections. Vero cell surface was commonly characterized by peripheral flat and central microvillous surfaces, whereas in monolayers, neighboring cells with the flat periphery and the microvillous surface frequently confronted each other (Fig. 5). It seemed that the flat surface of one cell (right side in Fig. 5) was covered with the flat surface of the other (leftside). No particle similar to the C type virions on L cell surface was encountered in HeLa and Vero cells. WI-38 cells (Fig. 6) and human skin fibroblasts (Fig. 7) possessed a distinctive morphology of their surfaces where microvilli were scarcely encountered. Cells frequently overlapped each other and thinly elongated projections and fibers, which seemed fibronectin filaments, extended widely between cells and were found between cells and the coverslip. The finest fibronectin filament was about 80Å in diameter, indicating the higher resolution of this technique. Morphology of both cells, WI-38 cells and human skin fibroblasts demonstrated here were common prior to their aging (unpublished data).

## DISCUSSION

The surface replica technique was originally introduced by Smith and Revel[4] into the analysis of concanavalin A binding sites on the surface of several types of animal cells. The cells were fixed doubly in 1.25% glutaraldehyde and 0.5% osmium tetroxide, dehydrated and dried in a stream of hot air after soakage in amyl acetate. Birdwell *et al.*[5] applied this technique but using the critical point drying technique to observe the assembly sites of Sindbis virus virions on the host cell surface. They doubly fixed the cells with 2.5%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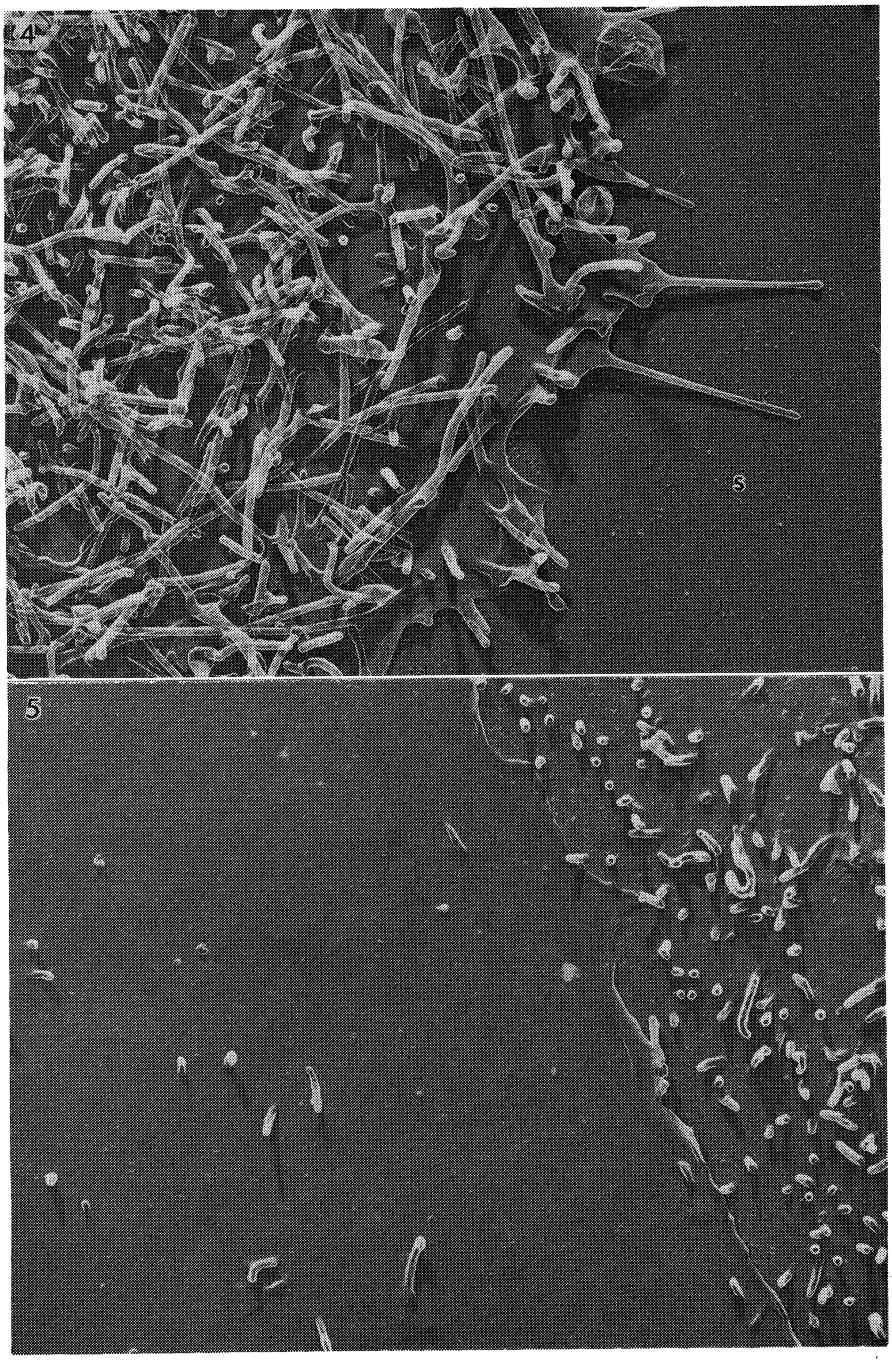
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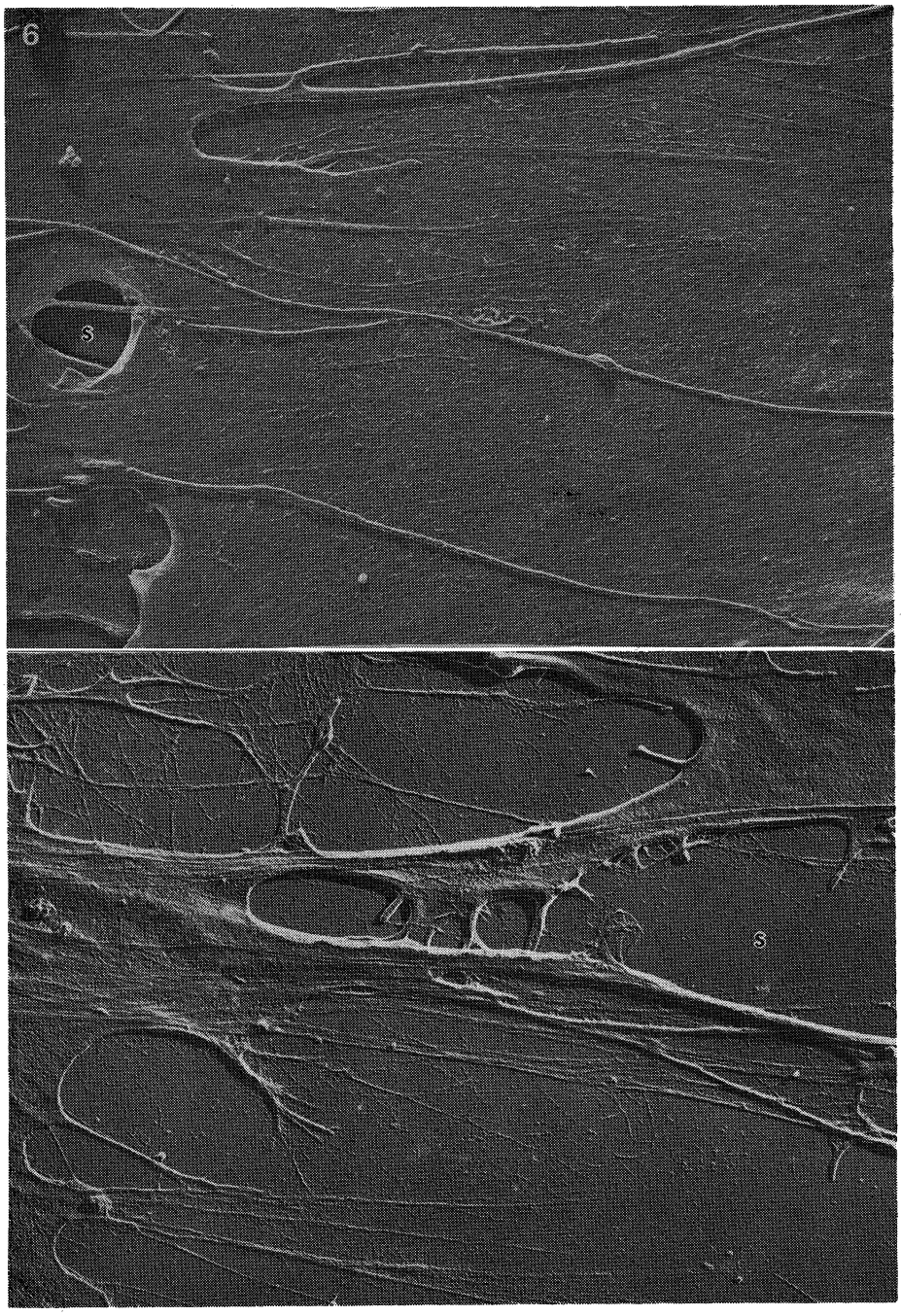
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Fig. 2. Surface replica image of L cell. Inset shows spontaneous C type virions. Letters s and scale bars in all figures indicate the surface of coverslip and 1 $\mu$m, respectively.
Fig. 3. A stereopair of peripheral region of L cell.
Fig. 4. Surface replica image of HeLa cell.
Fig. 5. Surface replica image of Vero cells.
Fig. 6. Surface replica image of WI-38 cells.
Fig. 7. Surface replica image of human skin fibroblasts.

glutaraldehyde and 0.5% osmium tetroxide in 0.1 M cacodylate buffer solution. The granularity of cell surface in the micrographs shown in both reports were seemingly rougher than those in the present report, suggesting the difference resulted from the concentration of fixatives used. Whereas no detail of shadowcassing was described in both reports, evaporation of metal and carbon should be carefully carried out to protect the specimen from the damage effect of radiant heat. In the present experiment the tungsten basket was heated up to 20 A of electric current for 5 sec at $1 \times 10^{-6}$ Torr and 25–30 A for 60 sec for carbon coating. The results indicated that the evaporation was enough to form shadow in good contrast and the smoothness of cell surface was well preserved under such conditions. The thickness of carbon coating was sufficient to protect the replica membrane from mechanical agitation during washing. However, some difficulties in cleaning of the replica membrane were occurred when carbon coating was 300 Å and more in thickness. Examinations of many microvilli and thinly elongated projections indicated complete removal of fixed cell component, suggesting that the cell component was dissolved and leaked out through the replica membrane. This might be the reason why the surface replica technique was applicable to many types of cells without reference to their complication of the surface morphology. The results obtained from the stereoscopy, three dimensional observations were readily carried out by this techinque. The higher resolution of the replica technique indicated that the fine markers, such as ferritin and hemocyanin, might be readily introduced into this technique for the immunological and cytological studies of cell surface activities.

**Acknowledgment**

The authors wish to thank Drs. T. Kimoto and M. Namba, and Miss A. Miyoshi for supply of Vero, WI-38 cells and human skin fibroblasts.

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