
# MICROSCOPY, BARCELONA 2001

Joint XX Meeting of the Spanish Microscopy Society,
V Meeting of the French Microscopy Society
co-ordiated with the
French Near-Field Microscopy Association,
XXXVI Meeting of the Portuguese Society
for Electron Microscopy and Cell Biology,
co-ordiated with the
IX Meeting of the Spanish Society for Cell Biology

## Barcelona, 3 - 7 September, 2001

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# FIXATION FOR FREEZE FRACTURE OF STRATUM CORNEUM: CHEMICAL FIXATION AND PROPANE-JET OR HIGH PRESSURE METHODOLOGIES?

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## INTRODUCTION

The outermost layer of mammalian epidermis, the stratum corneum (SC), consists of thin keratinized cells (corneocytes) embedded in a lipid-enriched intercellular matrix organized in lamellae. The main function of the SC, the barrier function, is probably located in the specific structure of this tissue. Thus, a number of skin studies are based on visualization techniques [1]. One problem of these studies is the immobilization of the SC structure for electron microscopy.

Cryotechniques have now proved to be the best method to prevent drying artefacts in the study of biological materials. However, a control of the freezing is necessary to minimize the formation of ice crystal that could damage the samples. In order to avoid this problem, sufficiently high cooling rates have to be used. Chemical fixation followed by propane-jet freezing and high-pressure freezing (HPF) are presently well-established techniques [2]. HPF is faster and allows to immobilize thicker samples. However, the freezing quality is variable and depends critically on the water content. In fact, for very thin samples with low water content (such as the SC) the chemical fixation followed by propane freezing has also resulted appropriate.

In this work a comparison between HPF of chemically untreated SC samples and propane-jet freezing of chemically fixed SC samples was established.

## MATERIALS AND METHODS

The epidermis was removed from pig skin with incubation with water at 70° for 4-5 min and after were placed in 0.5% trypsin in PBS at 4° overnight, and 2 h more at 25° in fresh 0.5% trypsin [3]. After several washes the SC pieces were fixed by two different methods:

1. High pressure freezing fixation (HPF) without chemical fixation using a Balzers equipment (temperature: -196°C of $LN_2$, pressure: 2100 bars) [4].

2. Chemical fixation using 2% glutaraldehyde in 0.1% cacodylate, overnight, at 4°C, followed by cryoprotection with 30% glycerol in 0.1% cacodylate, 1h at 25°C. After this the samples were frozen using liquid propane immersion at -190°C .

All frozen samples were freeze-fractured and coated with platinum-carbon using a freeze-etching unit BAF 060 (BAL-TEC, Liechtenstein). Fracturing was carried out at 150°C with the microtome method, in a $10^{-7}$ mbar of vacuum. An unidirectional shadowing of the fractured surface was made by evaporating 2 nm platinum-carbon at an angle of 45° followed by 20 nm of carbon evaporated at a 90° angle.

High resolution low temperature SEM

The replicas were immediately cryo-transferred on a Gatan cryo-holder into a Hitachi S-900, in lens field emission scanning electron microscope equipped with a highly sensitive annular YAG-detector for back scattered electrons [6]. Specimens were investigated at -110°C without any digestion, directly frozen.The beam current was $1\text{-}3x10^{-11}$ A as measured with a Faraday cage. The primary accelerating voltage was 10 kV. Images were obtained with the back scattered electron signal and recorded digitally with a Gatan Digiscan 688 connected to an Apple Quadra 950 [4] .

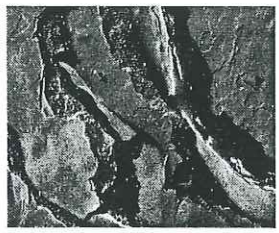
Room temperature TEM

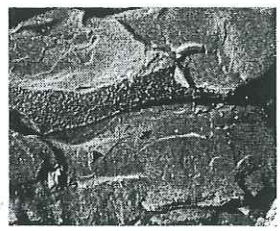
The replicas for transmission electron microscope were cleaned with an acid mixture (acetic acid, nitric acid and ortophosphoric acid) for 3 days, followed by 5% sodium hypochlorite for 1 day and several washes in distilled water and then picked up on Formvar-coated grids. The replicas were observed at room temperature using a Hitachi 800 MT at 75 kv.

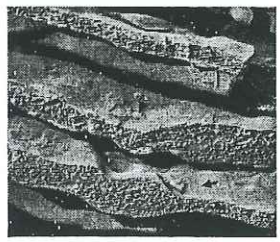
## RESULTS AND DISCUSSION

Our results indicate that although both freezing techniques were appropriate for the study of the SC ultrastructure, the plane of freeze-fracture was different depending on the fixation and freezing methodology used.

### CHEMICAL FIXATION AND PROPANE-JET FREEZING



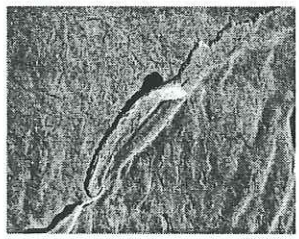




### HRLTSEM MICROGRAPHS

These micrographs show flat and smooth surfaces (fractures along the lipid lamellae, arrows ↘), sharps steps (fractures across lipid lamellae, arrowheads ▶) and granular surfaces, characteristic of the keratin filaments due to fracture across the corneocytes (C). Given these images we can assume that the fracture plane did not show preference to a specific way. A possible fracture plane is showed in the following representative cartoon:

### HIGH PRESSURE FREEZING FIXATION



### HRLTSEM MICROGRAPHS

In these pictures it can be observed that the fracture plane run mainly between the lipid lamellae. As a consequence, the micrographs show in most cases flat and smooth surfaces corresponding to the fracture between the lipid lamellae (arrow). This phenomenon was observed in HRLTSEM observations without removal of the biological material (micrographs ) and in TEM observations (micrographs ), where replicas were completely cleaned.

### TEM MICROGRAPHS

## CONCLUSIONS

These results seem indicate that the HPF preserves the natural behaviour of the SC, which has a tendency to be fractured along the weaker areas, that is, along the lipid lamellae. Propane-jet freezing of chemically fixed samples, on the other hand, provides a more homogeneous fracture behaviour. Thus, depending on the methodology used, we can favour a visualization of either lipid or protein domains of the SC. These results could be very useful in future ultrastructural studies in order to facilitate the microscopical visualization and interpretation of the complex images such as those of SC and even of other samples in which different domains coexist.

## REFERENCES

Van den Bergh et al, 1999, J Control Rel, 62, 367-379

Leforestier et al ,1996, J Micros , 184, 4-13

López O., De la Maza A., Coderch L. and Parra J.L., 1997, Colloids Surfaces A, 123-124, 415-424.

Walther P., Wehrli E., Hermann R. and Müller M., 1995 , J Microsc, 179, 229-237.

# Fixation for freeze fracture of stratum corneum: chemical fixation and propane-jet or high pressure methodologies?

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The outermost layer of mammalian epidermis, the stratum corneum (SC), consists of thin keratinized cells (corneocytes) embedded in a lipid-enriched intercellular matrix organized in lamellae. The main function of the SC, the barrier function, is probably located in the specific structure of this tissue. Thus, a number of skin studies are based on visualization techniques (Van den Bergh et al, 1999, J Control Rel, 62, 367-379). One problem of these studies is the immobilization of the SC structure for electron microscopy. Cryotechniques have now proved to be the best method to prevent drying artefacts in the study of biological materials. However, a control of the freezing is necessary to minimize the formation of ice crystal that could damage the samples. In order to avoid this problem, sufficiently high cooling rates have to be used. Chemical fixation followed by propane-jet freezing and high-pressure freezing (HPF) are presently well-established techniques (Leforestier et al,1996, J Micros, 184, 4-13). HPF is faster and allows to immobilize thicker samples. However, the freezing quality is variable and depends critically on the water content. In fact, for very thin samples with low water content (such as the SC) the chemical fixation followed by propane freezing has also given appropriate results. In this work a comparison between HPF of chemically untreated SC samples and propane-jet freezing of chemically fixed SC samples was established. The frozen samples were freeze-fractured, coated with Pt/C and transferred to a scanning electron microscope under liquid nitrogen and imaged on a cryo-holder at temperatures of about 113 K or digested and observed in a transmission electron microscope. Our results indicate that although both freezing techniques were appropriate for the study of the SC ultrastructure, the plane of freeze-fracture was different depending on the fixation and freezing methodology used. In the samples frozen by HPF without chemical fixation, the fracture plane laid mainly between the lipid lamellae. As a consequence, the micrographs showed in most cases flat and smooth surfaces corresponding to the fracture between the lipid lamellae (*figure 1*, arrow). However, when chemi-

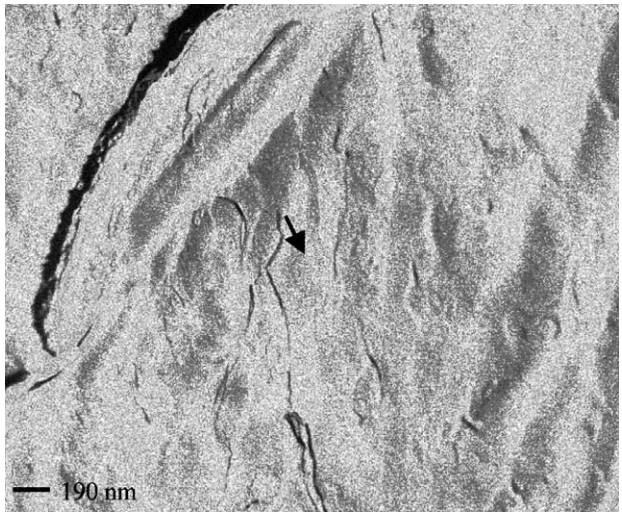


**Figure 1.** Micrograph of stratum corneum frozen by HPF without chemical fixation.

cal fixation and propane-jet freezing was used, the fracture plane did not show preference to a specific way. These micrographs showed flat and smooth surfaces (fractures along the lipid lamellae, see *figure 2* arrow), sharps steps (fractures across lipid lamellae, see *figure 2* arrowhead) and granular surfaces, characteristic of the keratin filaments due to fractures across the corneocytes (*figure 2*, open arrow). These results seem to indicate that the HPF preserves the natural behaviour of the SC, which has a tendency to be fractured along the weaker areas, that is, along the lipid lamellae. Propane-jet freezing of chemically fixed samples, on the other hand, provides a more homogeneous fracture behaviour. Thus, depending on the methodology used, we can favour a visualization of either lipid or protein domains of the SC. These results could be very useful in future ultrastructural studies in order to facilitate the microscopical visualization and interpretation of the complex images such as those of SC and even of other samples in which different domains coexist.


**Figure 2.** Micrograph of chemically fixed and propane-jet frozen stratum corneum.