Variation of enamel microstructure in vertical Hunter-Schreger bands

（縦型ハンター・シュレーゲル条における種々のエナメル質構造）

Tomohiko Hirayama

Department of Histology, Nihon University School of Dentistry at Matsudo

日本大学　松戸歯学部　組織学講座

平山友彦

（指導：岡田裕之　教授）

Preface

This article is based on reference papers, "Undulating Vertical Prism Decussation of *Pyrotherium* (Pyrotheria, Mammalia) Molar" and "Vertical Prism Decussation of *Teleoceras* (Rhinocerotidae) Molar" in International Journal of Oral-Medical Science.

Abstract

Mammalian teeth are covered with enamel, and tooth morphology is determined by the species' phylogenetic background and the manner of mastication and occlusion. The internal structure of enamel also exhibits characteristic microstructures, such as enamel prisms and Hunter-Schreger bands (HSB). In this study, the structure of two fossil mammalian molars, exhibiting a rare form of vertical HSB (unlike the horizontal HSB normally found in mammalian enamel), were compared. Additionally, the common structure and diversity of the vertical HSB were investigated. Two specimens were used in this study: *Pyrotherium*, an extinct mammal that lived in the Late Oligocene (about 25 million years ago) of South America; and *Teleoceras*, an extinct rhinoceros species from the Late Miocene (about 10 million years ago). In both mammals, the enamel on the occlusal surface of the molars showed fine ridges radiating from the inner to the outer surface.

In order to make the features of HSB easier to analyse, a sample for scanning electron microscopy (SEM) spattered with gold-palladium (Au-Pd) was observed with a stereomicroscope using reflected light. In addition to the observation of the polished plane, natural fractures were examined to confirm the twisting of the enamel prisms by SEM.

Three-dimensional reconstructions obtained by serial SEM images of tangential section visualized the change of movement and the positional relation of enamel prisms. These results indicate that there is a significant difference in regularity between the two vertical HSBs. In *Teleoceras*, the HSB zone is clearly divided, with a row of enamel prisms acting as the boundary, while in *Pyrotherium*, the HSB zone was elongated with various widths, extending in the tooth axis direction; the boundary of HSB was formed by the difference in the inclination of enamel prisms and indistinct dendritic striae connected the wide main regions of HSB. In *Pyrotherium*, there were also several deformations on the cross-sectional shape of enamel prisms. The three-dimensional reconstruction clarified the position and shape of enamel prisms in regions at different depths, twisting and undulation were both present in enamel prisms of *Pyrotherium* and *Teleoceras*, and the relative positional relation of adjoining enamel prisms also changed. However, the change in the shape and the course of the enamel prism in *Pyrotherium* molars were more irregular and frequent, respectively, than those in *Teleoceras*. The minute structures of the occlusal surfaces in the molar enamel were similar in the two mammal groups, which were not phylogenically related and the external morphology of the molars. Although the dynamics of the enamel prisms are different, the existence of a common character of HSB could be regarded as an example of convergent evolution in the histological structure.

Introduction

The shape of enamel prisms and Hunter-Schreger bands (HSB) varies among mammalian taxa. HSB in the enamel structure of the cheek teeth (i.e., molars and premolars) can be used to distinguish between the orders of mammals [1, 2] and recent studies have classified various HSBs [3-5]. Previous studies report that HSBs in rhinocerotoid [6-11] and *Pyrotherium* cheek teeth run vertically [4], whereas horizontal HSB is normally predominant in mammals. This study used the molars of *Pyrotherium* and rhinocerotoid *Teleoceras* as comparative experimental material to examine vertical HSBs. *Pyrotherium* was a large ungulate with an estimated body weight of 3.5 tons, living in the Oligocene (about 23-28 million years before the present: Ma) of South America [12-14]. Their cheek teeth were bilophodont with two transverse lophs [12, 15] (Fig. 1). The phylogeny and ecology of *Pyrotherium* still remains unclear to this day, due to the scarcity of remains discovered. *Teleoceras* lived in North America during the late Miocene and Pliocene (about 16-20 Ma), its body size was similar to that of the hippopotamus and its molars were rectangular, with two consecutive L-shaped lophs [21] (Fig. 2). The microstructure of the molar enamel was observed using scanning electron microscopy (SEM) and supplemented by three-dimensional images based on serial SEM images.

Materials and Methods

Materials

Molar fragments of *Pyrotherium* from the Department of Histology (Anatomy II), Nihon University School of Dentistry at Matsudo, Japan (MDH–no. 01998) and fragments

of *Teleoceras* molars from the Thomas Burke Memorial Washington State Museum in Seattle (USA) (UWBM no.61586) were examined. Additionally, a molar of *Teleoceras* (a private collection, SKPC #0014) from the Bone Valley Formation in Florida was observed.

Some fragments were embedded in polyethylene resin and sectioned tangentially (parallel to the dentinoenamel junction (DEJ) or outer enamel surface (OES)), longitudinally (radial to the tooth axis) and horizontally (perpendicular to the tooth axis). After polishing and slight etching with 0.05 N HCl, the specimens were sputter-coated with Au-Pd.

Light microscopy analysis

The specimens were analyzed under a reflected light microscope (SZ; Olympus Corp., Tokyo, Japan). The etching of the polished enamel surface provided a delicate morphology. When the light source was moved from one side to the other, the appearance of the surface changed with the orientation and the angle of the prisms.

Scanning electron microscopy analysis

To investigate the microstructure of the molar enamel, polished and fractured sections were examined with SEM (S2700 and S3400N; Hitachi Ltd., Tokyo, Japan) using secondary or backscattered electron methods depending on the specific conditions. To determine the three-dimensional course and profile of the enamel prisms, sections were consecutively ground down in 10 μm steps tangentially to the DEJ, up to a depth of 50 μm.

Three-dimensional reconstructions

The identification of the successive images of prisms was simplified by creating holes as location marks on the enamel surface using an Er: YAG laser (Erwin Advel; Morita

Corporation, Tokyo, Japan). These holes remained in a constant position throughout the series of sections. The location marks enabled the registration of successive tracings by adjusting the function of Photoshop (Adobe Systems Incorporated, San Jose, CA, USA) using serial SEM images. Separate tracings of each successive outline of 10 adjacent prisms were then made on Photoshop layers and the outlines of corresponding prisms were identified, numbered, traced, and digitized using a graphics tablet (Intuos 3; Wacom, Saitama, Japan). Three-dimensional reconstructions were produced with the "OZ" image rendering software (Rise Corporation, Sendai, Japan). In these reconstructions, each prism was shown in a different color and could be viewed from any perspective.

Results

The thickness of *Pyrotherium* molar enamel varied between the mesial and distal margins of the lophs. The thin margin of the enamel formed comb-like fine ridges (Fig. 3), which were continuous with the lateral surface of the molars. Loose wavy extensions on the lateral surface along the tooth axis were observed (Fig. 4), as well as undulating bands elongated with various widths extending in the tooth axis direction and irregular dendritic streaks along the bands in the tangential section (Fig. 5). In the tangential SEM image of the indistinct boundary area, the cross-sectional form of the enamel prisms gradually changed and prisms appeared to be fused and collapsed in some parts (Fig. 6). In the densely packed regions of the boundary, various orientations and deformed shape of enamel prisms with overlapping flattened morphology were observed (Fig. 7).

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Observation of the *Teleoceras* molars using the reflected light microscope revealed that the radial ridges came up to the occlusal enamel (Fig. 8). The polishing surface for SEM showed vertical decussation of enamel prisms along the tooth axis, while some bands bifurcated and discontinued their course on the tangential section (Fig. 9). In the SEM image of the tangential sections, the arrangement of enamel prisms in each band appeared to be in the opposite direction recognized by the oblique-cut surface. One or two lines of enamel prisms, which appeared as a cross-cut surface, formed the border between the bands. The boundary enamel prisms were compressed in rounder and more irregular shapes than the non-boundary enamel prisms (Fig. 10). In the horizontal sections, enamel prism direction was slightly different between the bands, and the boundaries between the bands appeared as longitudinal cutting surfaces of enamel prisms (Fig. 11). In SEM images of a longitudinal section of a natural fracture, the enamel prisms were intricately intertwined and each of prisms twisted on itself. As a result, its positional relationship changed with the adjacent prisms (Fig. 12).

Three-dimensional reconstruction from continuously SEM images of *Pyrotherium* molar enamel showed convoluted enamel prisms at boundary site (Fig. 13). The letters A-K indicate individual enamel prisms, which are assigned different colors. The start and endpoints of the same enamel prisms are indicated by A-A' to K-K'. Changes in the shape and relative position of the enamel prisms were remarkable, and there was a substantial twist within the enamel prisms. In the three-dimensional visualization of the *Teleoceras* specimen, the group of prisms was viewed from the upper-left oblique, and each prism appeared in a different color for the same letter (A-A' et al.; Fig. 14). Prisms A-G and A'-G'

were all located in the same plane. Inclinations and orientation changed the shape of prisms as the surface was ground off in the stages of depths from A-G to A'-G'. This reconstruction revealed a displacement of position and shape in areas of different depths.

Discussion

Most mammalian teeth are covered by enamel and the external tooth shape is determined by species' phylogenic background and their methods of mastication and occlusion. Histological structures such as HSB and enamel prisms play an important role in supporting the external structure of the tooth; now considered as standards for the classification of enamel.

Pyrotherium, one of the specimens observed in this study, is an extinct mammal that lived in the Oligocene (about 23-28 million years before the present: Ma) of South America. The other specimen, *Teleoceras*, is an extinct rhinoceros species from the late Miocene and Pliocene (about 16-20 Ma). The two species are completely independent of each other in terms of area, era, and lineage.

In both species, the enamel on the occlusal surface of the molars showed fine ridges radiating from the DEJ to the outer surface (Figs. 3, 8). In the tangential section, HSB formed a thick line extending in the tooth axis direction and the dendritic structure was confirmed in *Pyrotherium* molar enamel, and which was classified into vertical HSB observed in the rhinoceros including *Teleoceras* (Figs.5, 9). In this study, the relationship between the structure of two fossil mammalian molar enamel in which a very rare vertical

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HSB (unlike the horizontal HSB found in almost all mammalian enamel) was compared, and the common structure and diversity of the vertical HSB were investigated.

In the tangential section of the *Pyrotherium* molar enamel, a thick and wavy trunk extending in the direction of the tooth axis with dendritic streaks was observed (classified as vertical HSB), although it is very different from typical rhinoceroid molar enamel [6, 7, 10]. The boundaries of each HSB band in *Pyrotherium* is less clearly defined, where enamel prisms with very different inclinations come into contact or where the inclination rapidly changes. The cutting feature in tangential sections of the boundary prisms is either arcade or oval, but it was disintegrating the shape in place and more irregular and deformed than in the non-boundary prisms (Figs. 8, 9). The prisms seem to be pressured and twisted from the circumference. On the other hand, the rows of the prisms belonging to each HSB band in *Teleoceras* appear to run in the opposite direction in the tangential sections, and one or two lines of prisms formed the boundary between the bands (Fig.10). The enamel prisms of the fracture surface showed twists on each axis and bending, and the positional relationship between the prisms appeared to change (Fig.12). The obtained image of prism groups shows that the prisms change their vicinity or position and bend, twist, and/or cross with each other.

The reconstructed image shows that each prism changes its shape and is distorted along the path (Figs. 13,14). Enamel prisms run continuously through the DEJ to the enamel surface, forming external tooth morphology unique to each species. However, the reconstructed image of the *Pyrotherium* molar boundary region is so complex that it is difficult to follow the orientation of individual enamel prisms. It is particularly complicated

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compared to all 3D images of vertical HSBs published so far [22]. The place where the boundary is indistinct is where the inclinations of the enamel prisms change more gradually. The fact that the cross-sectional shape of the enamel prisms is significantly deformed and appears to be flattened indicates that a rapid change in orientation and direction occurred. The prism of the fracture surfaces corresponds to the three-dimensional image (Fig.12), indicating changes in the respective directions and locality relations of prisms along their rows. It could, therefore, be inferred that the prisms in the boundary area become torsional or disordered during amelogenesis. Unlike most other mammals, rhinocerotoids and pyrotheria had acquired the ability of ameloblast to form independently the vertical HSB in their phylogeny. HSB indirectly displays the course of enamel prisms and the three-dimensional motion of ameloblasts; moreover, it can indicate the formation process of the external form [11, 23-28]. Thus, the significant radial ridges observed at the occlusal surface (Fig. 3,8) possibly result from the vertical decussation attributed to chewing fine food in the rhinocerotoids lineage. These differences can be explained by the fact that enamel structure is the basis of enamel morphology and reflects tooth function, while HSB is a structure involved in the resistance to tooth fracture [29, 30]. However, it is necessary to consider HSB in terms of its relationship with tooth adaptation for food habit and mastication.

In this study, the minute structures of the occlusal surfaces of the molar enamel of two extinct mammal groups were shown to be similar, despite the species not being related phylogenetically and the different external morphology of the molars observed. Although the dynamics of the enamel prisms between the specimens differed, the existence of vertical

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HSB in both is regarded as an example of convergent evolution in the histological structure of the tooth.

Conflicts of interest

The authors have no potential conflicts of interest.

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Figures and legends

Fig. 1 Skull of the *Pyrotherium*. (a) Lateral view (b) Palatal view (From Carroll R L, 1988).

Cheek teeth (i.e., molars and premolars) are bilophodont with two transverse lophs, in which enamel elliptically surrounds dentin by attrition.

Fig. 2 Cheek teeth of the *Teleoceras*. (From Cook HJ, 1927).

Cheek teeth (upper rigft molars - premolars) are angular shape with enamel lophs surrounding dentin.

Fig. 3 Thin margin of the enamel. Note the comb-like ridges. Scale bar = 1 mm.

Fig. 4   Lateral surface of the molar. Note the wavy extension along the tooth axis.

Scale bar = 1 μm.

Fig.5 Tangential section observed by reflected light microscope. Streaks of irregular width were lined up loosely along the tooth axis. Threadlike side branches connected the thick streaks. Scale bar = 500μm.

Fig.6 Tangential section (SEM). Enlarging the region of the clear boundary. Prisms with different orientations meet at the boundary.

Fig.7 Tangential section (SEM). Note the various orientations and deformed sections of enamel prisms.

Fig.8 Tangential surfaces of the molar under a reflective light microscope.

Scale bar =1 mm. E, enamel; D, dentin

Fig.9 Tangential surfaces of the molar under a reflective light microscope.

Scale bar =500μ.

Fig.10 SEM image of a tangential section (backscatter mode). One or two lines of prisms compose the boundary between the bands.

Fig.11 SEM observation of a horizontal section. The boundary is composed of the longitudinal cutting surfaces of prisms. The enamel surface direction is toward the top.

Fig.12 Changes in the prism direction and distortions observed in this broken longitudinal section. Scale bar = 15 μm.

Fig.13 Three-dimensional visualization of *Pyrotherium* molar enamel, the group of the prisms viewed from the upper left oblique. Each prism appeared in a different color with the same letter (A-A' et al.). The A-K shows the bottom and A'-K' shows the surface of a serial cutting plane, respectively.
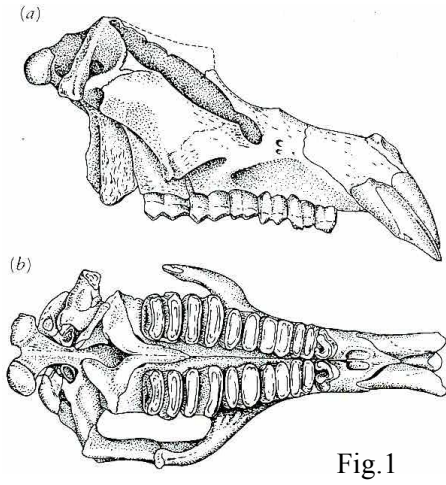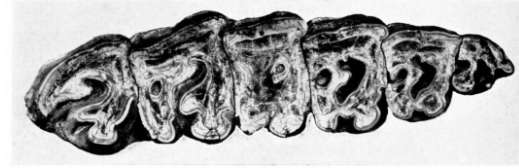
Fig.14 Three-dimensional visualization of *Teleoceras* molar enamel, of the group of the prisms viewed from the upper left oblique. Each prism appeared in a different color with the same letter (A-A' et al.). The A-G shows the surface and A'-G' shows the bottom of a serial cutting plane, respectively.

Fig.1

TELEOCERAS HICKSI.
Upper right molar—premolar teeth. Referred specimen No. 251, Colo. Mus. Nat. Hist. Approx. X½.
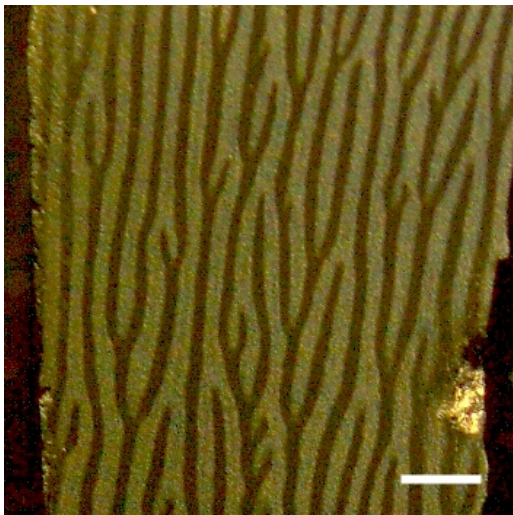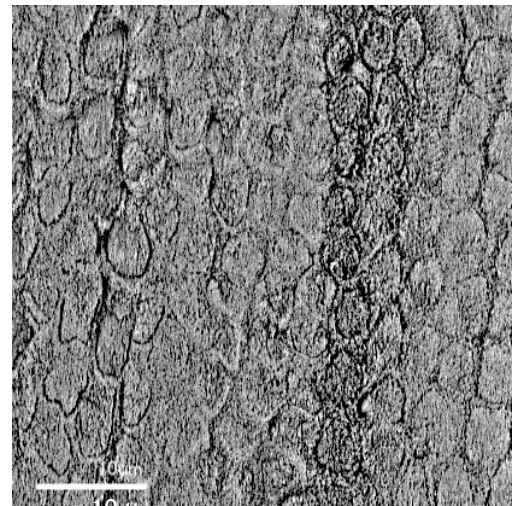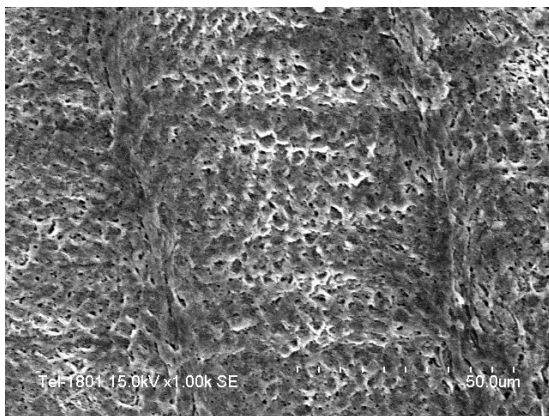
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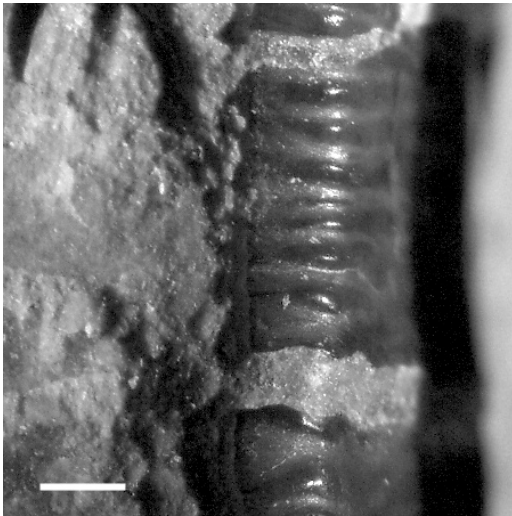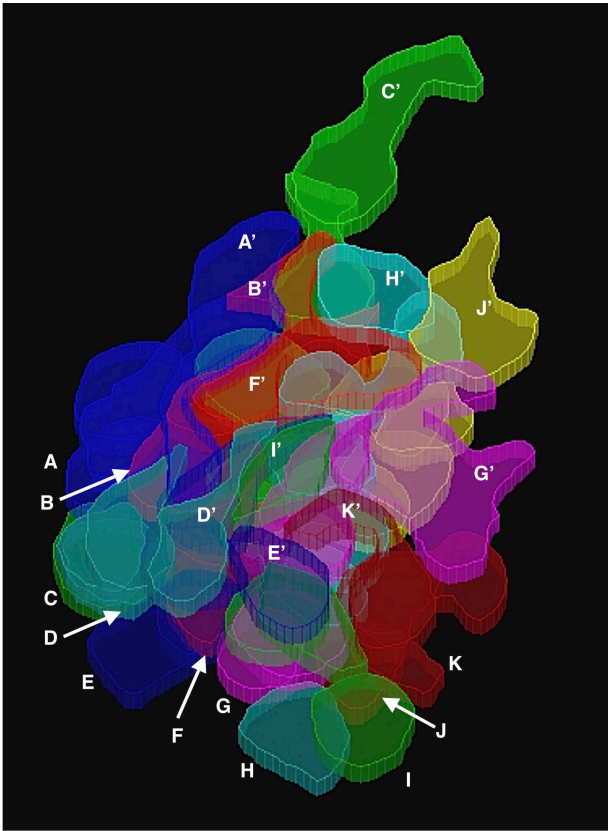

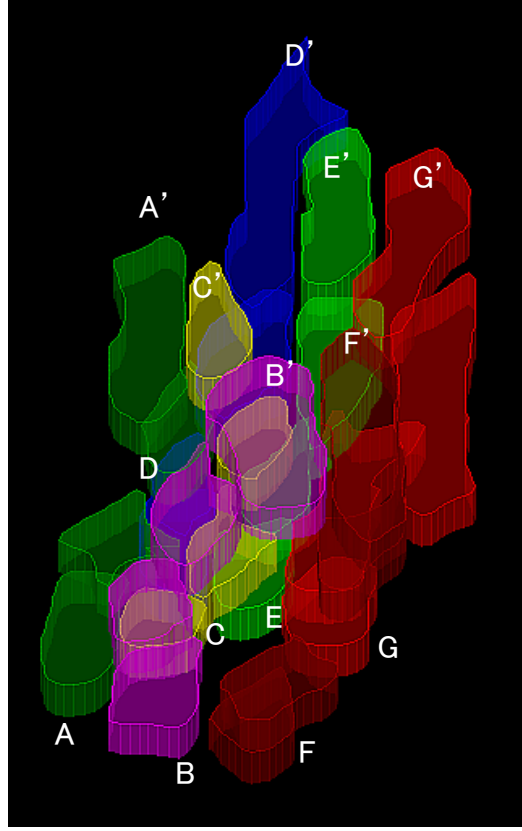Fig.3

Fig.4

Fig.5

Fig.6

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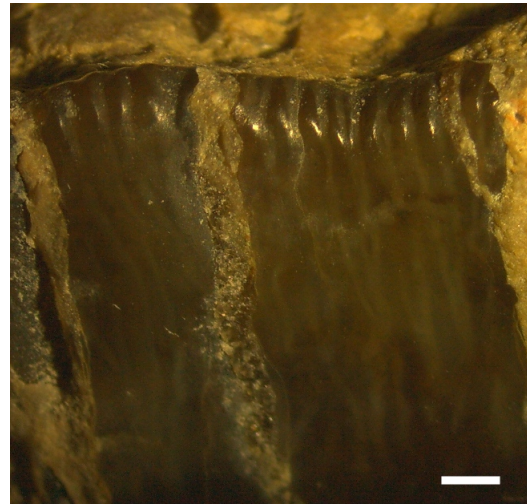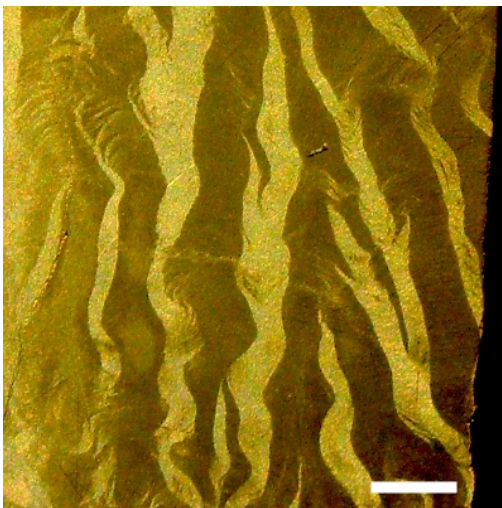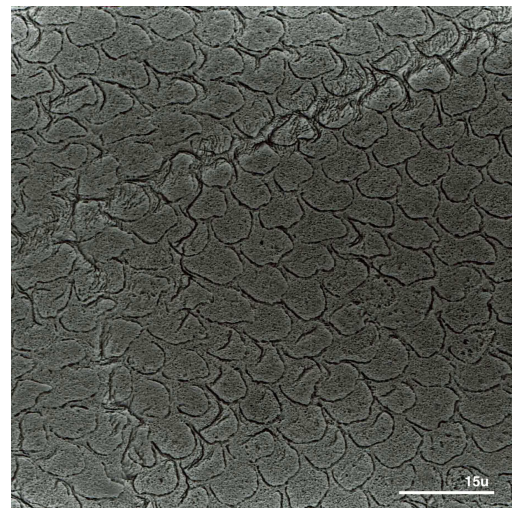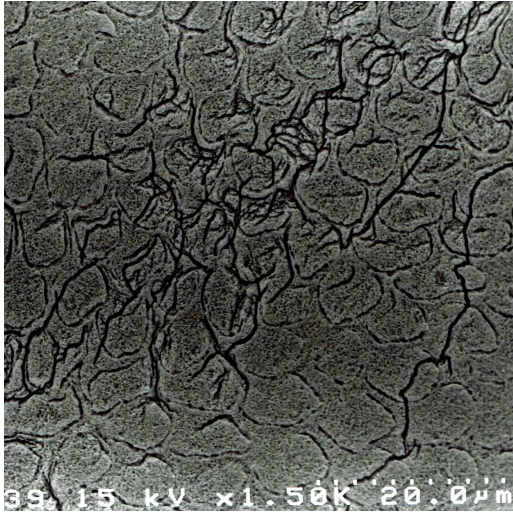
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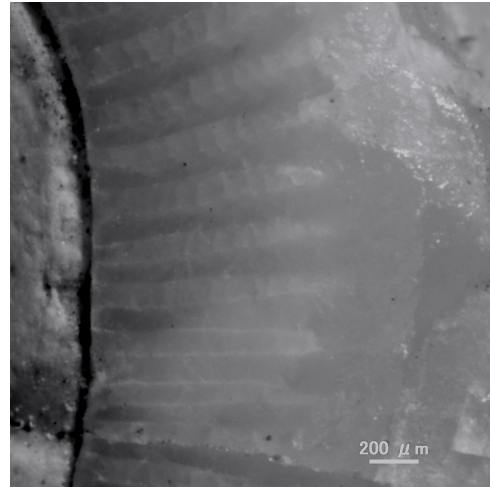
Fig.8

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