SHEET NO. 18

INSTRUMENT: MODEL HU-12A ELECTRON MICROSCOPE

SUBJECT: EFFECT OF USING MODEL HK-6 LARGE ANGLE TILTING DEVICE IN OBSERVATIONS OF BIOLOGICAL SPECIMENS

PREFACE

One of the things which surprises us in observing micrographs is that the pictures show a uniform sharpness and distinction over the entire range even when a specimen having a topographical surface profile is observed.

This is one of the features of electron microscopes having a great depth of focus, but it may also be said a serious defect of such electron microscopes since the difference in height on the specimen surface, that is, stereo-structures of the specimen, cannot be read out from the micrographs.

When we try reading the stereo-structure of the specimen there is no other way but to imagine the structure from our past experiences while observing a micrograph or to assume it from several micrographs of the serial ultra-thin sections.

At any rate, such work is very tedious and difficult in most cases.

To examine stereo-structures of specimens, the shadowing technique and negative staining technique have conventionally been employed because of easy specimen preparation.

However, they often encounter difficulties when the specimen has a topographic profile or the internal structure of the specimen is examined stereoscopically. Consequently, they are applicable to some limited specimens only (such as bacillus, vivus, etc.). The stereoscopic observation is very useful in such a case.

The following description deals with the principle and various requirements for stereoscopic observation, introduction of the Model HK-6 Large Angle Tilting Device, and several applications using this device.

Fig. 1 shows an external view of the Model HU-12A incorporated with the Model HK-6 accessory.

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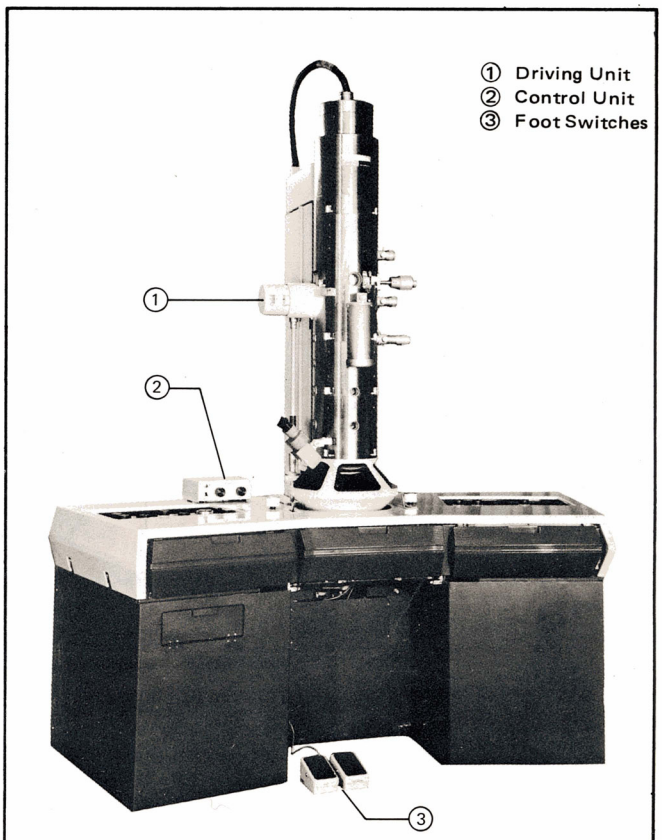


Fig. 1 Model HU-12A Electron Microscope incorporated with Model HK-6 Large Angle Tilting Device

1. PRINCIPLE OF STEREOSCOPIC OBSERVATION

The principle of the stereoscopic observation with electron microscopes is just the same as the stereoscopic observation and/or photographing with cameras. Any substance can be observed stereoscopically as far as we see it with both eyes.

In this case, a three-dimensional feeling is obtained since the viewing direction slightly differs between both eyes.

In order to obtain stereoscopic micrographs with an electron microscope, therefore, a picture should be taken at a desired visual field first, and then another picture of the same visual field should be taken with the same magnification after tilting the specimen several degrees. Now, a stereoscopic image of the micrograph can be viewed by observing the two pictures arranged in parallel through a stereoscope viewer.

The principle diagram of the stereoscopic observation on micrograph is given in Fig. 2.

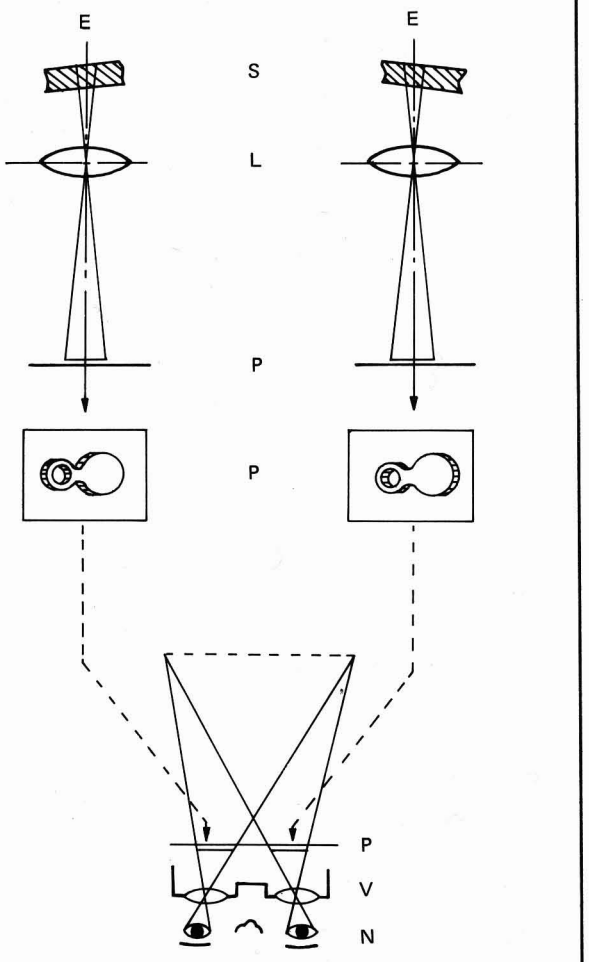


Fig. 2 Principle diagram of stereoscopic observation in electron microscope

2. REQUIREMENTS ON STEREO-MICROGRAPHY

Now, let's discuss various requirements on an ideal stereoscopic observation since desirable stereoscopic observations cannot be attained simply by taking two photographs with the specimen tilted. Various conditions such as the specimen thickness, tilt angle, etc. should be taken into consideration according to the penetrating efficiency of the electron beam.

a) IRRADIATION ANGLE AND DEPTH OF FOCUS

Let's take the conditions for electron micrography from the conditions in the case of stereoscopic observation with the naked eye.

According to Helmcke's experiments in 1954, man gets tired unless the difference of the visual angle between the right and left eyes is less than 70' when he gazes at an object with the naked eyes.

When the above value is converted into the depth of the object situated at a distance allowing distinct vision, it is found that the object can be observed easily when the depth is about 20mm, provided that the distance between both human eyes is 65mm as a standard. (Refer to Fig. 3)

In other words, ideal observation can be done when the depth (thickness) of the specimen is about 20mm when observing two micrographs through a stereoscope. Next, let's examine the conditions for the electron micrography (See Fig. 4)

Assume that focusing is set at the bottom "a" of an ultra-thin section specimen having a thickness of "h". When point "b" is the focusing limit on the specimen, irradiation angle α (aperture angle of the electron beam to be irradiated) can be obtained by converting the blur at point "b" into that at point "a" and expressing it by $h \cdot \alpha$.

Since the resolution of the naked eye is 0.1mm, the image quality deteriorates to degrade the stereoscopic image if the blur at point "b" is noticeable with the naked eye.

Therefore, the blur degree at point "b" should be less than 0.1mm.

Also, when the stereoscopic image is formed at such magnification (20/h) that "h" is magnified to be just 20mm, the blur degree corresponding to $h \cdot \alpha$ should be less than 0.1mm as follows:

$$h \cdot \alpha \cdot 20/h \leqq 0.1$$
$$\alpha \leqq 5 \times 10^{-3} \text{ rad.}$$

In order to assure ideal stereoscopic observation, it is therefore necessary to take stereoscopic pictures on condition that the irradiation angle fully meets the above expression ($\alpha \leqq 5 \times 10^{-3}$ rad.).

b) MAGNIFICATION, SPECIMEN THICKNESS, AND TILT ANGLE OF SPECIMEN

One of the factors in determining how to select the magnification and the tilt angle of specimen in preparing stereoscopic pictures is the difference of visual angle. As described above, the difference of visual angle is 70' for ordinary people, and if it is less than 70', the stereoscopic feeling is reduced, causing the stereoscopic observation effect to decrease.

When a stereoscopic picture is observed through a stereo-viewer, the distance between the stereoscope lens and the stereoscopic picture is adjusted usually so that the

stereoscopic image is obtained at a distance allowing distinct vision.

Since the parallax corresponding to the difference of visual angle of 70' (1/50 rad.) under this condition becomes 250 x 1/50 = 5mm (where 250mm is the distance allowing distinct vision), it is necessary to prepare the stereoscopic pictures so that the parallax of the stereoscopic image becomes 5mm (See Fig. 3).

Also, the parallax of the stereoscopic image is determined according to the specimen thickness (depth), magnification (final magnification), and tilt angle of specimen. This can be expressed by the following equation according to Fig. 5.

$$p = M \cdot h \sin\theta$$

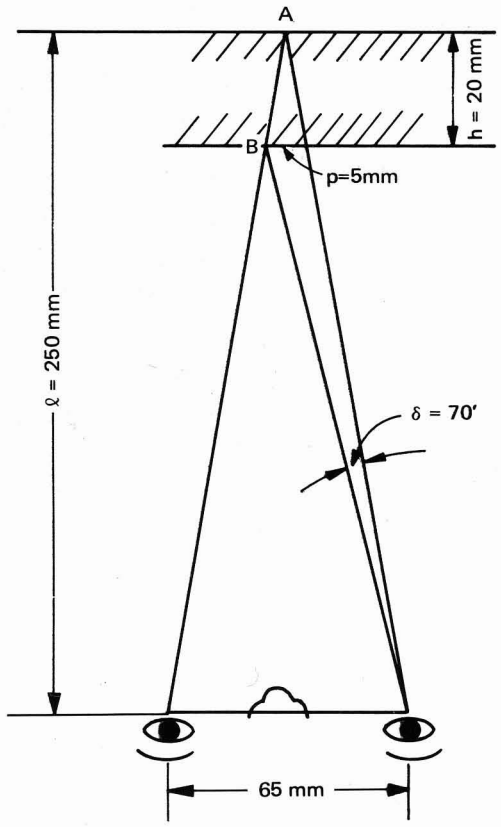
where p: Parallax (mm)
M: Magnification (final magnification: Magnification through stereoscope viewer)
h: Thickness of specimen
θ: Tilt angle of specimen

Thus, M and θ are selected from the above equation so that p becomes 5mm.
When $\theta = 15^\circ$, for example, magnification M becomes;

$$M = \frac{p}{h \cdot \sin\theta} = \frac{5}{h \times 0.26} \fallingdotseq \frac{19}{h}$$

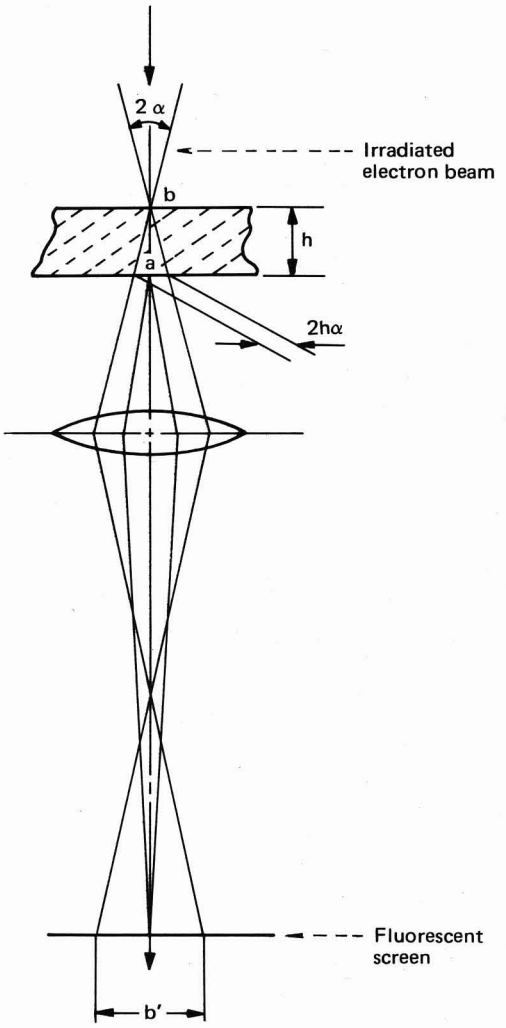
Therefore, if the specimen is 0.4μm in thickness, the magnification of an ideal stereoscopic image becomes;

$$19/h = \frac{19}{4 \times 10^{-4}} = 47{,}500X$$



h: Maximum depth of object (thickness of specimen in stereoscopic observation)
p: Parallax $\left(250 \times \frac{1}{50} = 5\text{mm}\right)$
δ: Difference of visual angle $\left(70' = \frac{1}{50}\text{ rad}\right)$
ℓ: Distance allowing distinct vision (250mm)

Fig. 3 Limit of visual field depth at distance allowing distinct vision



α: Aperture angle of electron beam which irradiates onto specimen
h: Thickness of specimen
a: Point in focus
b: Limit of focused range when point "a" is in focus.
b': Blur degree of image at point "b"

Fig. 4 Depth of focus in electron microscope

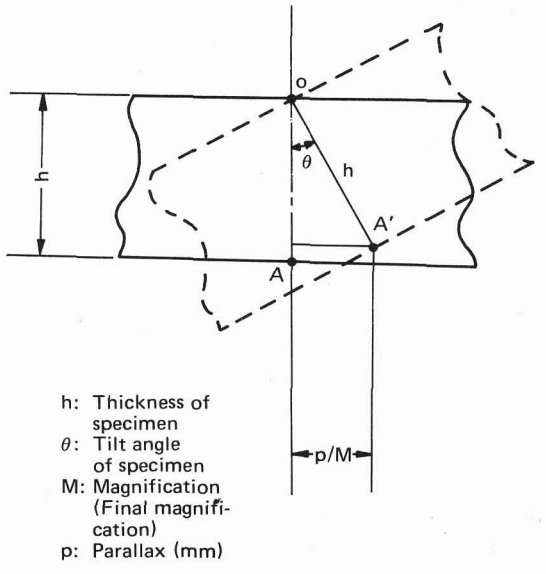


Fig. 5 Relation among the tilt angle of specimen, parallax, and thickness of specimen

In addition, if the magnification of stereo-viewer is 2X, the magnification of the stereoscopic picture should be $\frac{47,500}{2} =$ 23,800X.

Since the above description deals with an ideal stereoscopic observation, such a strict magnification is not always required but it should be close to the above equation according to the purpose when taking pictures.

However, be careful since image trouble may occur when the specimen is tilted largely. Our experiences reveal that the limit of tilt angle of the specimen is 10° to 15° for the purpose of observing stereoscopic pictures.

c) DIRECTION OF SPECIMEN TILTING AXIS

The specimen tilting axis means a fulcrum axis for the specimen tilting. It should be particularly noted when preparing stereo-micrographs that the visual field be determined before photographing so that the specimen tilting axis which corresponds to the neutral line of a pair of stereo-micrographs meets the line vertical to the straight line which connects both human eyes. Because the electron microscope image rotates around the beam axis which is the center in the magnetic field of the lenses when the magnification varies, the direction of the specimen tilting axis at the final image also rotates when changing magnification. In the zoom lens system of the Model HU-12A Electron Microscope, rotation of the final image due to magnification change is reduced by compensating it through combination with reverse rotation of the intermediate lens and the first and second projection lenses.

However, since rotation of the final image is inevitable to some extent, it is recommended to determine the magnification which is used most frequently and set the azimuth of the tilting axis so that it meets the neutral line of the stereoscopic pictures.

In practical operation, it should be noted that the tilting azimuth illustrated in the tilting device is actually perpendicular to the tilting axis of the specimen. This will serve to reduce a possible error in interpreting pictures later.

d) EQUALIZING THE MAGNIFICATIONS OF A PAIR OF PICTURES

It is of course necessary for obtaining ideal stereo-micrographs that the magnification remain unchanged when taking a pair of pictures.

However, a specimen apart from the tilt axis may often be observed in the electron microscope.

In this case, the distance between the principal plane of the objective lens and the specimen changes.

Therefore, if focusing is done by adjusting the exciting current of the objective lens, the magnification varies correspondingly so that it must be corrected in the photographic enlargement process of the micrograph.

For a countermeasure, it is recommended that the specimen tilting stage be provided with a vertical control system of the specimen so as to compensate the specimen position according to tilt angle. Then, a pair of stereo-micrographs can be obtained with the magnification kept equal for the two pictures, thus assuring accurate analysis of pictures.

3. MODEL HK-6 LARGE ANGLE TILTING DEVICE

This device permits tilting the specimen at an desired azimuth within a range of 0° to ±35°.

The tilt and azimuth angles are controlled by the respective drive systems and can be changed by the two foot switches independently.

The tilt and azimuth changeover speed can be selected continuously by the knob on the control unit according to magnifications during observation.

The tilt and azimuth angles are indicated on the illuminated scale drum and can be read out even in a dark room.

For the purpose of observing medical or biological ultra-thin sections stereoscopically, the Model HK-6 permits tilting the specimen toward both the plus and minus sides from the horizontal position with the tilting azimuth after setting this azimuth in the direction of longer side of the film. Also, it permits compensating the specimen position change due to tilting by shifting up and down the entire specimen stage so that the focal distance of the objective lens can be kept constant.

Thus, the Model HK-6 has functions to meet the stereoscopic observation.

4. APPLICATIONS

Let's discuss the advantages in stereoscopic observations referring to several applications with the Model HK-6 Large Angle Tilting Device employed.

The following pictures were all taken at 125kV.

APPLICATION-1 Effect of thickness of ultra-thin section

Fig. 6 indicates the pictures of an ultra-thin section of

500Å thickness taken at a tilt angle of 0° and 40° for the purpose of comparison, and shows little change of villus membrane after tilting the specimen at 40° from 0°.

Fig. 7 gives pictures of a relatively thicker section of 0.4μm thickness taken at a tilt angle of 0°, 20°, and 35°, respectively. It shows a distinct state of growing villi and connection of cellular tissue.

It is therefore advantageous for the main purpose of stereoscopic observations of specimen to prepare a thick specimen. However, a staining technique should be carefully applied to the specimen with a high accelerating voltage imposed in this case.

For the purpose of observing fine microstructures with enhanced resolution, a thinner specimen is preferable.

APPLICATION-2 Effect obtained by photographing a visual field with a specimen tilted in all azimuths

Fig. 8 shows the pictures of an ultra-thin section of 5μm thickness taken at a tilt angle of 35° while changing the tilting azimuth in 45° steps.

Thus, the growing state of villi and the correlation between villus and cell can easily be observed stereoscopically from such an ultra-thin section by changing the tilting azimuth.

Particularly when a microtomic ultra-thin section is observed, various images indicating its oblique and/or longitudinal sections are obtainable depending upon the way it is cut. Accordingly, the change of the azimuth permits obtaining valuable pieces of information which may be unapproachable by conventional fixed observation methods.

APPLICATION-3 Calculation of thickness of ultra-thin section

Fig. 9 shows an example of obtaining a desired cross-sectional image by tilting the specimen at 35°. The image was blurred stream-like when the specimen was at the horizontal position (0°). From the two pictures given in this figure, the thickness of the ultra-thin section as well as the height or length of the tissue observed can be calculated.

Fig. 10 shows the typical drawing for calculation and the arrow (b) in Fig. 9 indicates the sample used for this calculation.

$$t = \ell \tan(90° - 35°) = 0.4\,(\mu m) \tan 55° \fallingdotseq 0.57\,\mu m$$

(ℓ: 0.4 μm)

$$L = \frac{\ell}{\sin 35°} = \frac{0.4(\mu m)}{\sin 35°} \fallingdotseq 0.698\,(\mu m)$$

Remarks:

A spiral substance is observed as shown by arrow (a) in the upper picture taken at a tilt angle of 0°. However, we are apt to overlook such a substance as a mere interposition when observing normal cross-sectional images as shown in the lower picture taken at a tilt angle of 35°.

From this fact, it may be said very effective to take a picture of a thick specimen with tilting observation.

The following two examples deal with actual researches.

APPLICATION-4 Fine microstructure of intramitochondrial inclusions appearing in epithelial cells of gill of hormomya mutabilis ... (1)

Fig. 11 shows pictures of epithelial cells of gill of hormomya mutabilis collected at Minamata bay in Kyushu, Japan. In picture (a), the surfaces of the epithelial cells are covered with many cillia and microvilli. Also, rootlets of cillia are distinctly observed, and many mitochondria, glycogen granules, and stratiform bodies exist in the proloplasm.

In some mitochondria, the existence of square or triangular dense inclusions is recognized, and this is the very organic mercury which has been regarded as the cause of the so-called Minamata disease according to a few researchers. (IRUKAYAMA et al (1961), and OURA (1972))

The picture (b) is an enlarged image of mitochondria including two inclusions. These inclusions may be classified into different substances or structures at first glance. One is dense lines buried in parallel, each line being 5nm in width and also 10nm in interval (from center to center), and the other is homogeneous dense ones.

The picture (c) indicates crystalline inclusions appearing in the matrix of mitochondria. These inclusions may be composed of dense lines arranged in parallel since a homogeneous dense structure as shown in (b) is obtained when the same inclusions are tilted by 20°.

Judging from the above observations, the crystalline inclusions which were thought at first glance to be two different substances, are the same substance as a matter of fact.

APPLICATION-5 Stereoscopic observation of Marek's disease of chicken and herpes virus of turkey ... (2)

The features of Marek's disease of chicken are tumescent multiplication of the lymphocyte in the peripheral nervous system, and internal organs and systems.

This disease is known to be caused by the herpes B group virus.

On the other hand, herpes virus of turkey (HVT) is separated from a normal and sound turkey, and its virusological and serological properties are analogue to those of Marek's virus (MDV). The HVT is applied as a vaccine since it gives no etiological disease to chickens.

According to the report of Prof. Fujimoto et al, Department of Veterinary Science, Hokkaido Univ., Japan, immature particles having various morphological features were detected in the nucleus when observing the MDV and HVT by means of electron microscopy after cultivating them with the renal cells of chicken (CKC) and the fibriform cells of quail (QF).

The three-dimensional structure of these immature particles was examined by using the Model HK-6 Large Angle Tilting Device as follows:

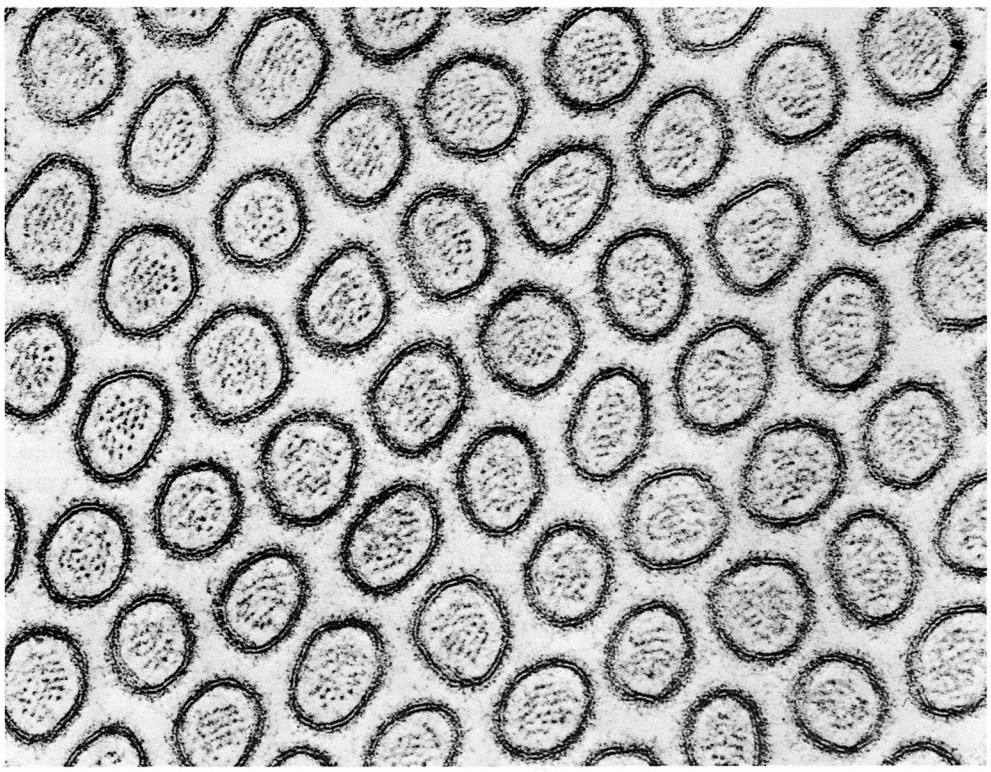
For confirming the immature particles having about seven different morphological structures detected in the nucleus through observation of the ultra-thin section, an ultra-thin section of about 0.5μm thickness was observed while tilting it at various angles and observed stereoscopically.

Fig. 12 shows an example of the above results.

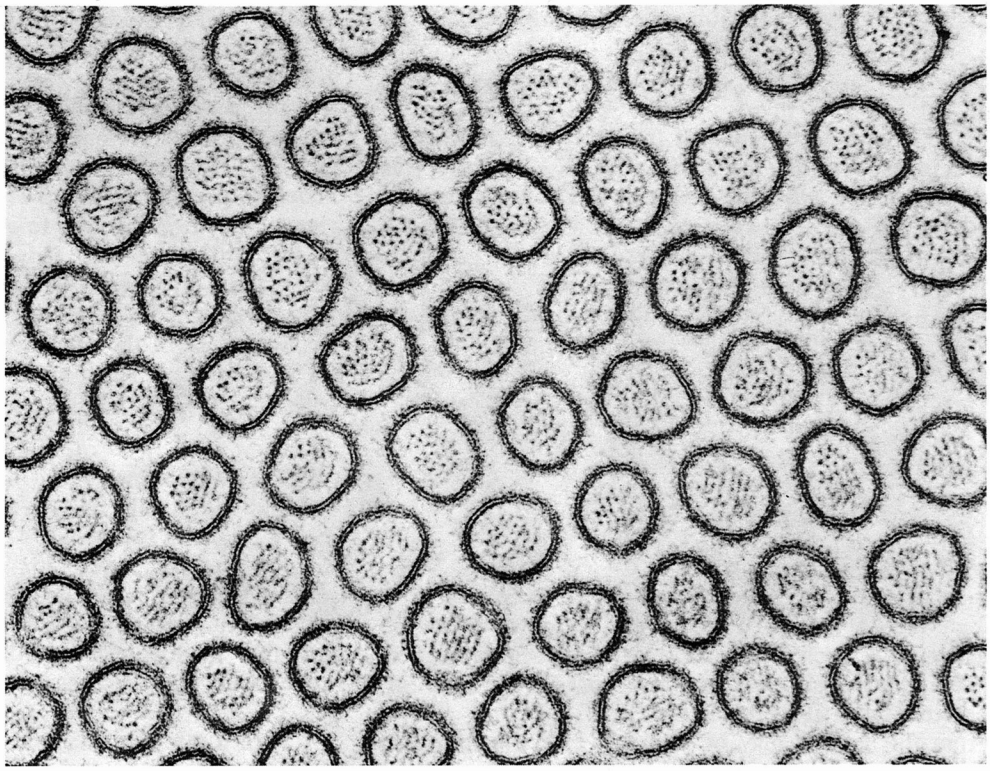
The observation using a stereo-viewer reveals that annular rings intersect each other at right angles in the capsid.

The bottom picture in Fig. 12 shows the model prepared according to the above results.

As described above, the stereoscopic observation permits application to virus or the like with ease.



Tilt 0°

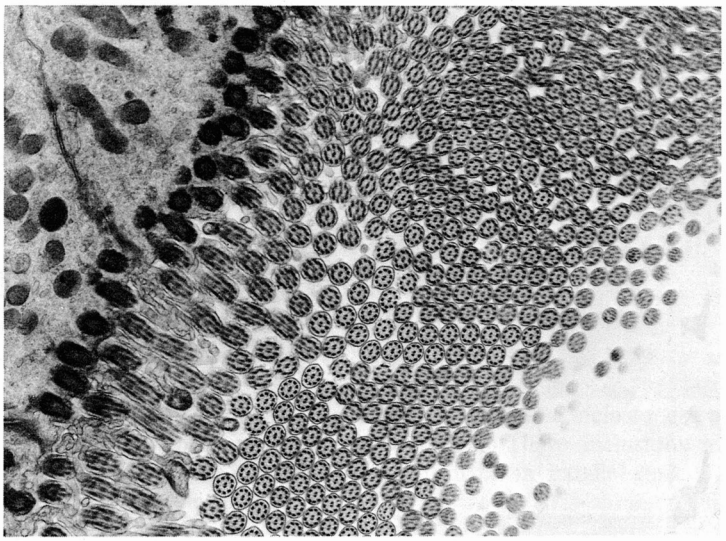


Tilt 40°

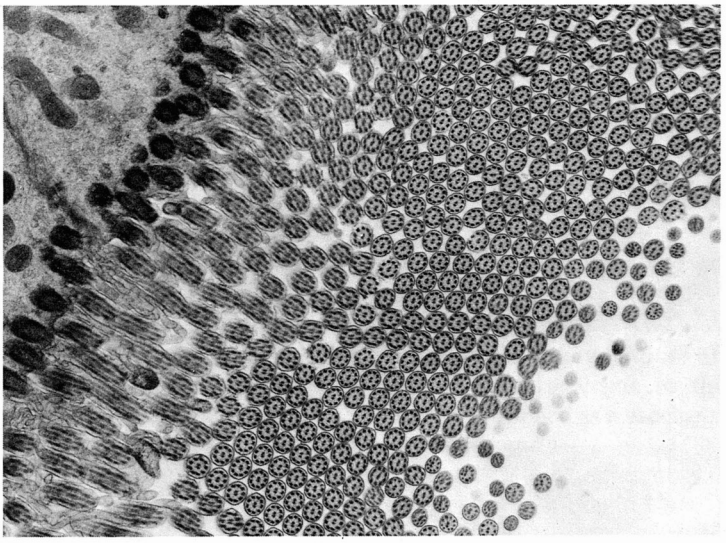
0.5 μm

Specimen: Microvilli of epithelial cells of mouse
(By the courtesy of Prof. Nagano, School of Medicine, Chiba University)
Thickness of specimen: 500Å

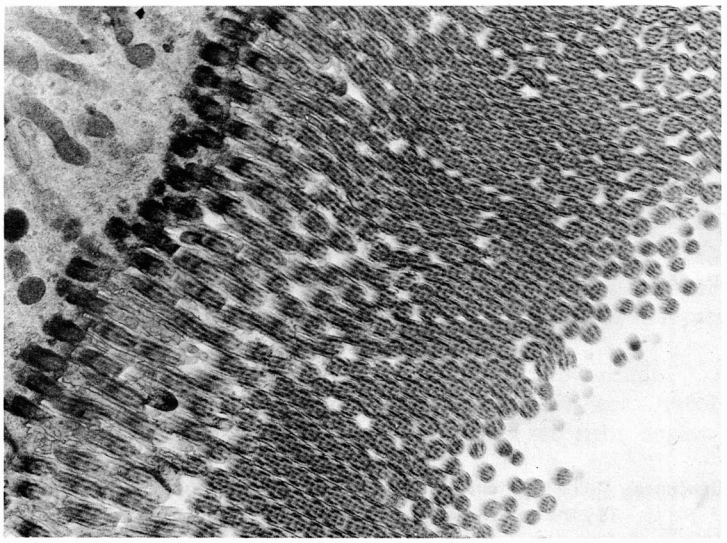
Fig. 6 APPLICATION-1



Tilt 0°



Tilt 20°

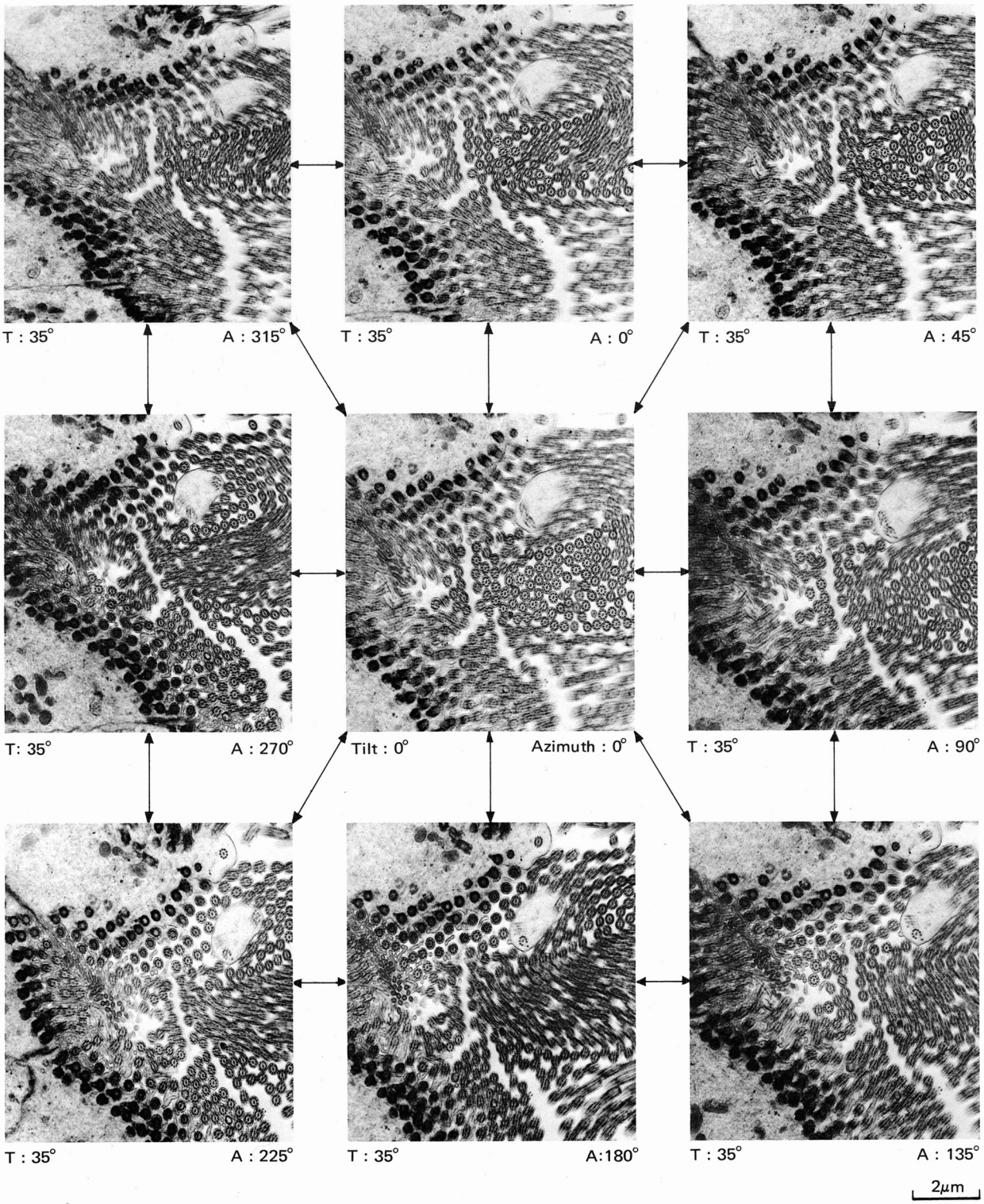


Tilt 35°

2μm

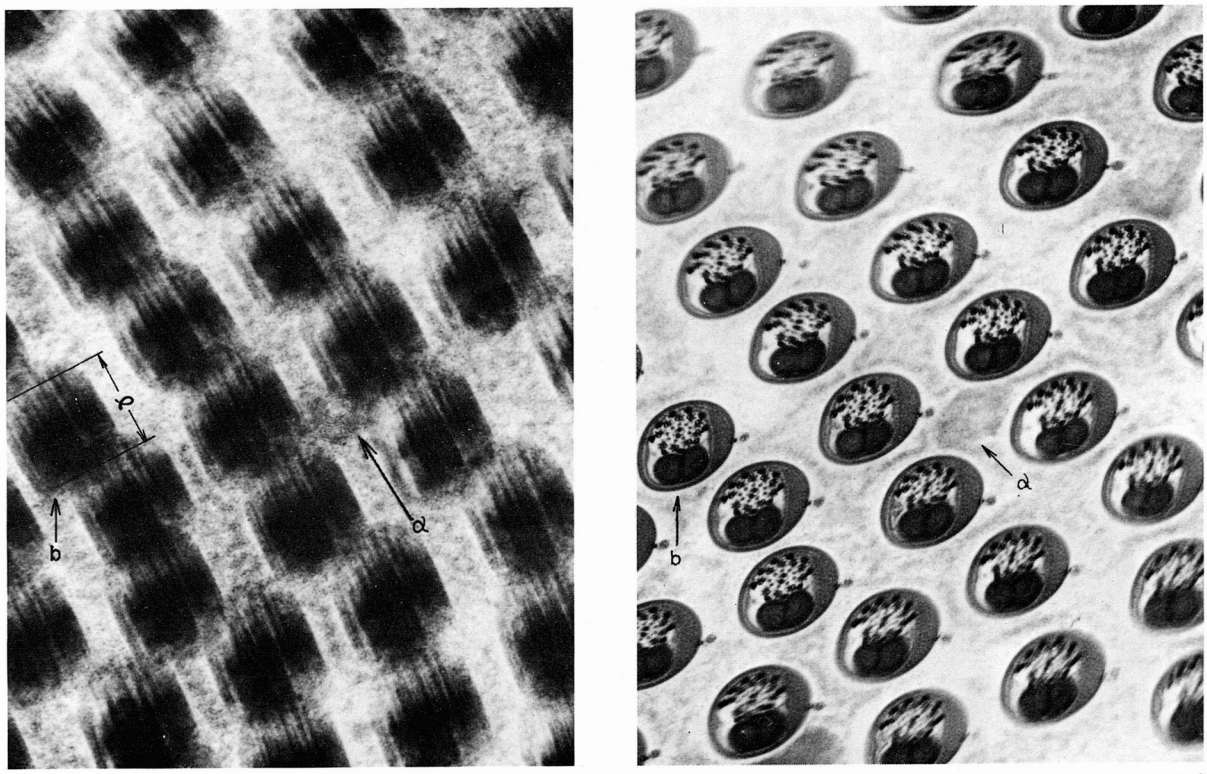
Specimen: Villi of epithelial cells of antrum highmori of rabbit
(By the courtesy of Prof. Fujimoto, Medical Department, Tokushima University)
Thickness of specimen: 0.4μm

Fig. 7 APPLICATION-1



Specimen: Villi of epithelial cells of antrum highmori of rabbit
(By the courtesy of Prof. Fujimoto, Medical Department, Tokushima University)
Thickness of specimen: 0.5μm

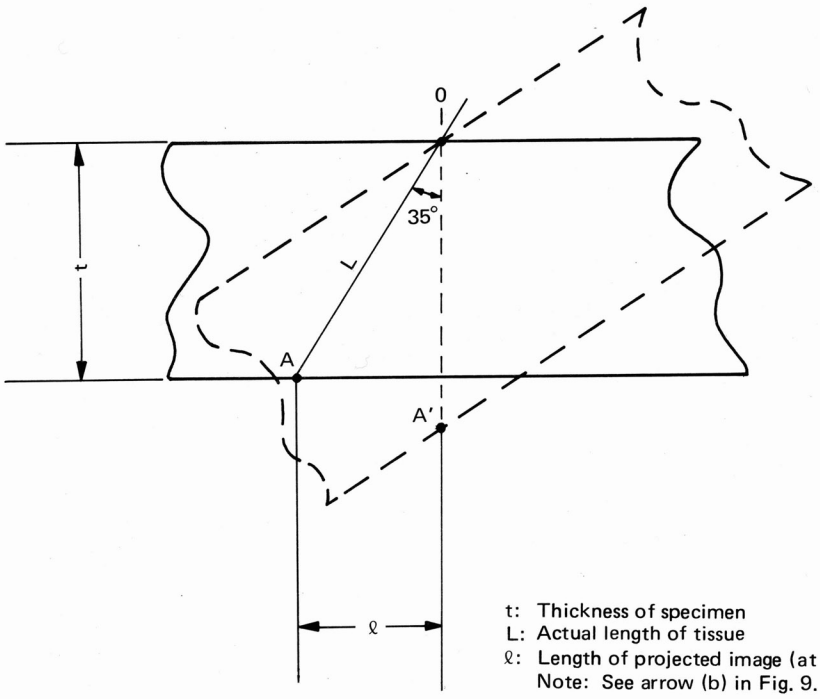
Fig. 8 APPLICATION-2



Tilt 0°　　Tilt 35°

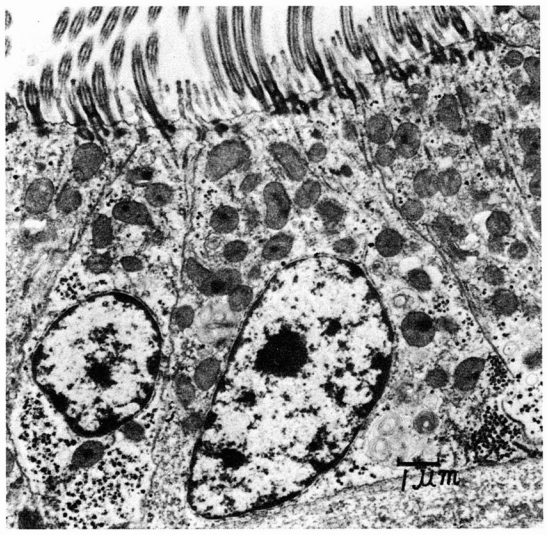
Specimen: Spermary of silkworm
(By the courtesy of Prof. Oura, School of Medicine, Kumamoto University)
Thickness of specimen: 0.57μm (calculated value, microtome scale: 0.5μm)

Fig. 9 APPLICATION-3

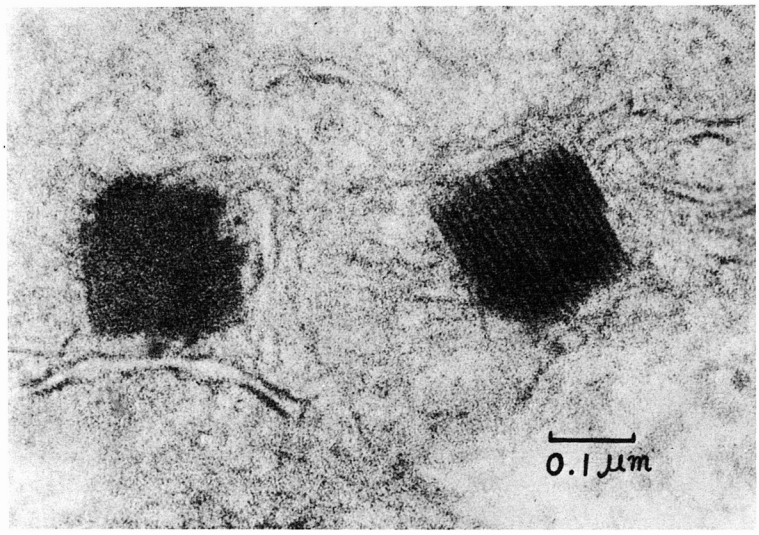


t: Thickness of specimen
L: Actual length of tissue
ℓ: Length of projected image (at tilt angle 0°)
Note: See arrow (b) in Fig. 9.

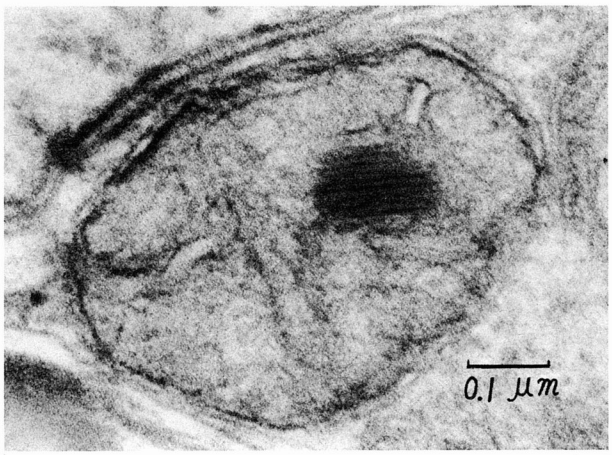
Fig. 10 Typical Drawing of Application-3



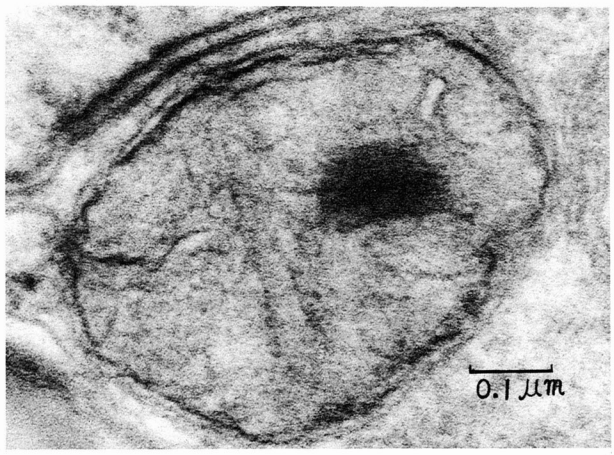
a



b



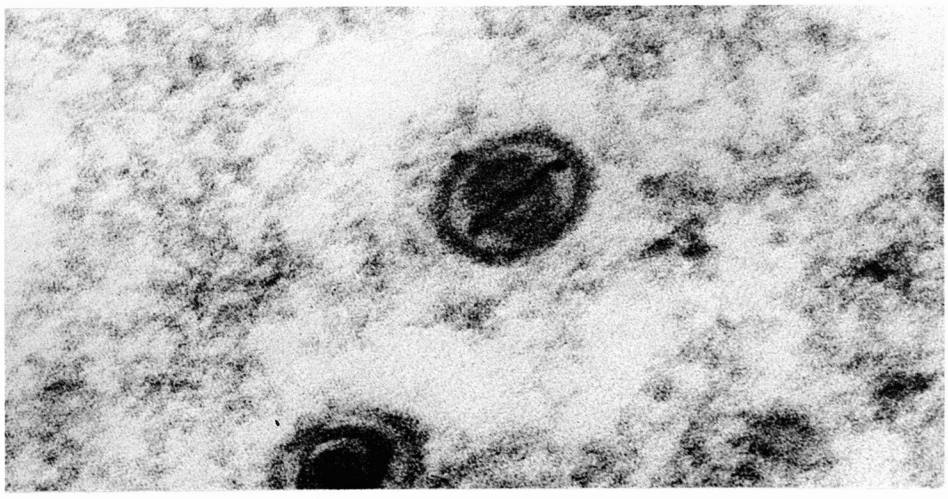
c Tilt 0°



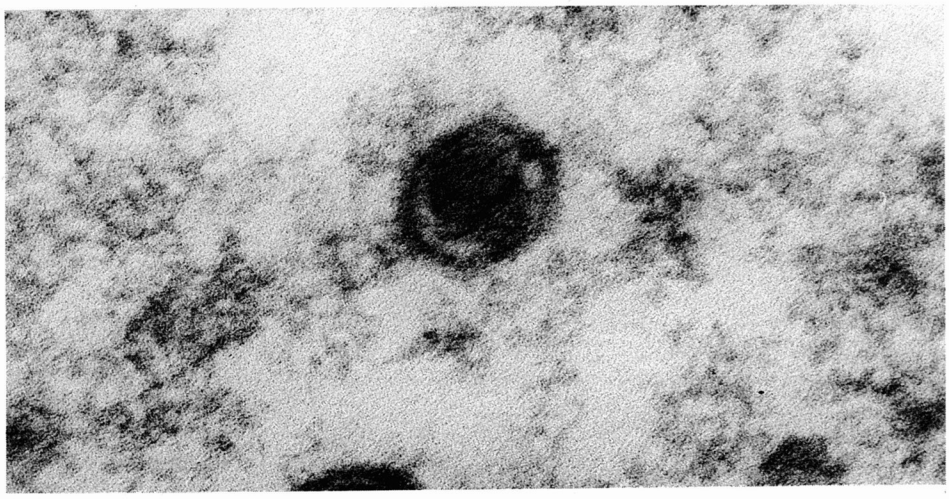
d Tilt +20°

Specimen: Hormomya mutabilis
(By the courtesy of Prof. Oura, School of Medicine, Kumamoto University)
Thickness of specimen: 0.1μm

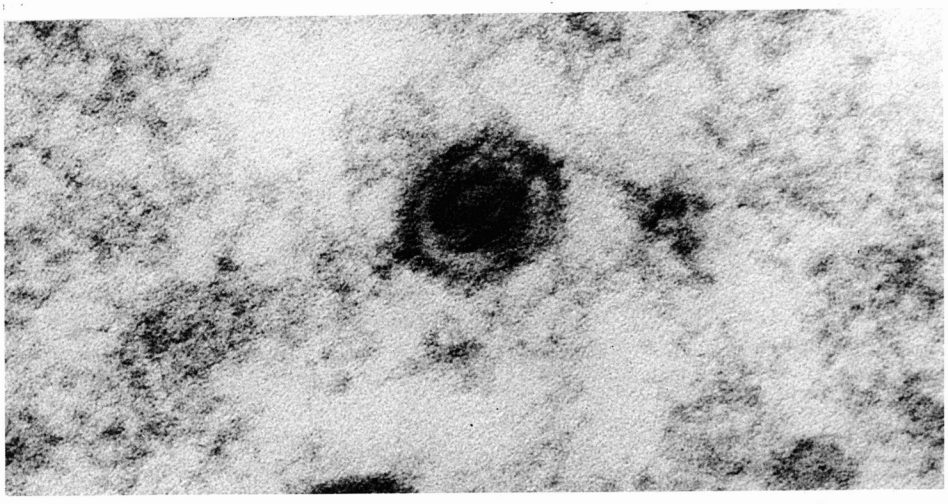
Fig. 11 APPLICATION-4



Tilt +35°

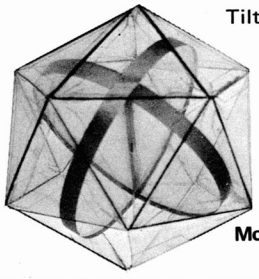


Tilt 0°



Tilt -20°

0.1μm



Model

Specimen: Nucleocapsid autopeculiar — shaped core
(By the courtesy of Profs. Fujimoto and Okada, Faculty of Veterinary Medicine, Hokkaido University)
Thickness of specimen: 0.5μm

Fig. 12 APPLICATION-5

5. SPECIFICATIONS OF MODEL HK-6

Tilt angle:	±35°
Azimuth:	360°
Resolution:	5Å (3.4Å guaranteed when Model HK-6 is delivered together with the Model HU-12A EM)
Magnification:	500X ~ 240,000X
Specimen grid:	Standard grid 3.2 mm dia.
Angle control system:	Motor-driven (with two foot switches)
Angle changeover speed:	Continuously variable from low to high speed (1 : 5)
Z-axis adjustment:	±0.5 mm

Reference
C. Oura, G. Yasuzumi, H. Akahori, K. Yonehara
The Fine Structure of Intramitochondrial Inclusions Appearing in Epithelial Cells of the Shellfish Gill
Monitore Zool. Ital. (N.S.) 6:147~153, 1972

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