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~~The distribution of the number of nuclei in teleost osteoclasts~~Odontoclasts in the Chinook salmon differ from mammalian odontoclasts by exhibiting a great proportion of cells with high nuclei number

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Abstract

Odontoclasts resorbing teeth are multinucleated cells. Previously, the authors have investigated the distribution of number of nuclei per human odontoclast, and showed that the mean number of nuclei per cell was 5.3 and median was 4 and 93.8% of cells had 10 or fewer nuclei. Teleost odontoclasts have features similar to those of mammals, however the distribution of number of nuclei per cell remains unknown. The present study aims to examine the distribution of number of nuclei per odontoclast in a teleost fish, Chinook salmon, *Oncorhynchus tshawytscha* (Walbaum), and to clarify the difference of number of nuclei in odontoclasts between Chinook salmon and humans. The maxillae and mandibles of Chinook salmon were fixed, decalcified, and embedded in Epon 812. Specimens were serially sectioned into 0.5-μm semithin sections, and examined by light microscopy. Cells possessing brush border adjacent to a resorptive lacuna were identified as odontoclasts, and 246 odontoclasts were investigated to determine the distribution of nuclei per cell. The mean number of nuclei per cell was 21.8, and median was 17; only 24.4 % of odontoclasts had 10 or fewer nuclei, and 95.5 % had 50 or fewer nuclei. These results suggest that the range of the number of nuclei per odontoclast in Chinook salmon is larger than that in humans.

Introduction

Odontoclasts resorbing teeth and osteoclasts resorbing bone have the common morphological features: multinucleation and the presence of a special structure termed ruffled border that can be seen on transmission electron microscopical pictures (light microscopic brush borders) (Scott and Pease, 1956; Marks and Popoff, 1988; Pierce et al., 1991). Many investigators examined the distribution of the number of nuclei per osteoclast or odontoclast, and have reported the relative frequencies of cells with 10 or fewer nuclei: 80% in human odontoclasts (Addison, 1978), 95% in kitten osteoclasts (Addison, 1979), 89% in rat osteoclasts (Ries and Gong, 1982), and 81% in chick osteoclasts (Piper et al., 1992). These reports disregarded the presence of mononuclear osteoclasts which were observed in serial sections based on criteria of both acid phosphatase activity and resorptive lacunae (Kaye, 1984) or odontoclasts because their presence was not proven precisely. Previously, we have demonstrated the presence of mononuclear osteoclasts with ruffled borders in vitro (Domon and Wakita, 1991) and mononuclear odontoclasts in vivo (Domon et al., 1994), and investigated the distribution of number of nuclei per human odontoclast, and reported that 93.8% of cells had 10 or fewer, including mononucleus (Domon et al., 1997). These reports indicate that the distribution of nuclei in osteoclasts or odontoclasts varies little in mammals.

In teleost fish osteoclasts are seen, and the appearance of odontoclasts associated with tooth replacement is well known. These teleost osteoclasts and odontoclasts have features similar to those of mammalian ones: multinucleation and the presence of ruffled borders (Berkovitz, 1977; Sire et al., 1990; Hughes et al., 1994; Huysseune and Sire, 1992, 1998). In mammals the parathyroid hormone (PTH) is one among other factors that control osteoclastic activity through osteoblasts, but PTH is not effective in teleost fish (Huysseune, 2000; Witten et al., 2001). This indicates that control of

osteoclastic resorption in teleost fish may be different from that in mammals. Several investigators examined the number of nuclei in teleost osteoclasts (Takagi et al., 1989; Witten, 1997; Witten et al., 2001), however the distribution of number of nuclei per odontoclast in teleost fish remains unknown. The present study aims to examine the distribution of the number of nuclei per odontoclast on shedding teeth of a teleost fish, Chinook salmon, *Oncorhynchus tshawytscha* (Walbaum), and to clarify the difference of number of nuclei in odontoclasts between Chinook salmon and humans.

Materials and Methods

Light and Transmission Electron Microscopy

Four Chinook salmon that were reared in fresh water at 10 °C, 145 - 165 mm S.L. (standard length), about 1 year age, provided through the courtesy of the Sapporo Salmon Museum (Sapporo, Japan), were used in this study. As animals did not reach sexual maturity, we did not check sex of them. Prior to decapitation, the animals were subjected to an overdose of MS222 (SIGMA, St. Louis, MO). After decapitation, the maxillae and mandibles were immediately fixed with 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.4) overnight at 4 °C, and then decalcified with 5% EDTA (pH 7.4) for two weeks at 4 °C. Specimens were postfixed with 1% osmium tetroxide in 0.05M sodium cacodylate buffer (pH 7.4) for 3 hr at room temperature. They were block-stained with 4% uranyl acetate for 30 min, dehydrated in a graded series of ethanol, and then embedded in Epon 812. The specimens were sectioned in the frontal direction (longitudinal to the axis of tooth) with diamond knives on a Reichert-Nissei ultramicrotome into 0.5-µm semithin or 0.1-µm ultrathin sections. The semithin sections were stained with toluidine blue, and observed with a light microscope (LM) (NIKON FX-A, Japan). Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a transmission electron microscope (TEM) (HITACHI H-7000) at an operating voltage of 75kV.

All animal experimentation followed the Guide for The Care and Use of Laboratory Animals, Hokkaido University Graduate School of Dental Medicine, which is based on the Guide for The Care and Use of Laboratory Animals (NIH, 1985).

Determination of the Number of Nuclei per Odontoclast

The determination of the number of nuclei per odontoclast followed the method of a previous report (Domon et al., 1997). To prevent the purposeful selection of specimens with specific regions seen to have large or small odontoclasts, three specimens were

chosen from among many Epon embedded ones at random. The specimens were serially sectioned in the frontal direction with diamond knives on a Reichert-Nissei ultramicrotome into 0.5-μm semithin sections. The sections were stained with toluidine blue, and cells associated with resorptive lacunae were photographed at a magnification of x 250 under an oil-immersion LM.

Cells with the following characteristics were identified as odontoclasts: presence of a light microscopic brush border adjacent to a resorptive lacuna, which is an apparent tiny pit or groove made on the dentine surface. The observations carefully established whether cells for investigation had resorptive lacuna under a brush border or not, and the cells which formed lacunae on the dentine or pedicel under brush borders, odontoclasts, were identified. The number of nuclei in the selected cells was counted on the serial sections. Odontoclasts were grouped according to the number of nuclei, and the fraction of the number of nuclei per odontoclast was calculated as a percentage of the total number of odontoclasts examined, and then a frequency table was produced. The cumulative frequencies of the number of nuclei per cell were recorded.

A Kruskal-Wallis test applied to the statistical analysis of the distributions of the number of nuclei per odontoclast among the three specimens selected at random. A Mann-Whitney U-test applied to the statistical analysis of the distribution of the number of nuclei per odontoclast between Chinook salmon and humans.

Three-Dimensional Reconstruction

Odontoclasts that were considered to be in the process of cell fusion, and were selected for further investigation. The photographs of the serial sections of these cells were enlarged on printing paper; and the cell surface, dentine surface, and nucleus were traced on tracing paper. Based on both the outline of the section and round cells which were identified as leukocytes and/or lymphocytes based on morphological criteria near the

dentine, these elements were input serially into a personal computer (NEC PC-9801 DX) with the Three-Dimensional Graphic Analytic System, COSMOZONE 2SA (NIKON, Japan). The reconstructed image was directly photographed from the computer display.

To determine accurately whether the investigated cells were fusing or not, both semithin sections of the investigated cells and the three-dimensionally reconstructed images were observed. The determination of cell fusion of odontoclasts followed the criterion of the previous report (Domon et al., 1998): there must be two or more nucleated cells in contact with each other at one site only in the three-dimensional reconstruction. Odontoclasts with more than 50 nuclei could not be reconstructed due to the memory limitations of the personal computer. In these cells, fusing cells were determined on the following criterion: two or more nucleated cells in contact with each other at one site from the observations of the serial sections.

Results

Most odontoclasts were observed in the dental pulp on the inner surfaces of shedding teeth and on pedicels, but cells were also seen on the outer surfaces of shedding teeth. The inner surfaces of shedding teeth were filled with numerous odontoclasts, and these odontoclasts were situated close to one another from the pedicel to the top of the pulp cavity (Fig. 1). Odontoclasts were situated on the resorptive lacunae of various sizes. The contours of the lacunae were wavy and some margins among lacunae were sharp, but most of the outlines of the resorbed surfaces were smooth. The frequency of such shedding teeth was only one per several functional teeth. In many functional teeth there were few odontoclasts.

The odontoclasts were multinucleated giant cells, and cells with 10 or more nuclei in one section were common (Fig. 2). Odontoclasts had various sizes and most were from 30 to 50 μm in diameter, cells of more than 100 μm diameter were often observed. Odontoclasts had brush borders, and formed resorptive lacunae on the dentine or pedicel under the brush borders. Osteoblasts and odontoblasts were not seen around resorbing odontoclasts on the dentine and pedicel surfaces. No deposition of bone-like tissue was observed on the resorbed surfaces of shedding teeth.

The TEM observations showed that odontoclasts had ruffled borders toward the resorptive lacunae on the dentine surfaces, and that the ruffled borders were composed of extensive membrane infoldings. (Fig. 3) Under the ruffled borders the collagen fibrils composing the dentine matrix were disrupted. Odontoclasts had many mitochondria and rough-surfaced endoplasmic reticulum in the cytoplasm, and there were many vacuoles in the cytoplasm near the ruffled borders.

Figure 4 shows serial semithin sections of an eighteen-nuclear odontoclast. This cell formed a resorptive lacuna on the dentine under the brush border. The cell was not in contact with other cells on the serial sections. The reconstructed image of this cell shows

that an odontoclast with rounded outline has eighteen nuclei scattered throughout the cytoplasm (Fig. 5). Other odontoclasts in this study showed similar three-dimensional images.

Figure 6 shows serial semithin sections of a thirteen-nuclear odontoclast and a seventeen-nuclear odontoclast. These two cells formed resorptive lacunae on the dentine surface under the brush borders, and they were in contact with each other at one site only. The three-dimensional reconstruction of the two cells in Figure 6 shows that the contact region on the dorsal surfaces of the adjacent cells formed a narrow bridge between the two cells (Fig. 7). The reconstructed image also shows that the nuclei were scattered throughout the cytoplasm of each cell. According to the criterion of cell fusion mentioned above, the two cells in Figure 6 were regarded to be in fusion and the cells were recorded as one cell with thirty nuclei. Using the criterion of cell fusion of the reconstructed images, 53 cells were founded to be in fusion. Cell fusion was observed between multinucleated odontoclasts as well as between multinucleated and mononuclear precursors (data not shown). Cell fusion was observed in cells which were adjacent to each other.

Odontoclast with more than 50 nuclei were observed on shedding teeth. Figure 8 shows serial semithin sections of an 89-nuclear odontoclast and a 58-nuclear odontoclast. These cells formed resorptive lacunae on the dentine surfaces, and had irregular-shaped cellular contours. These two cells were in contact with each other at one site from the observations of the serial sections. According to the criterion of cell fusion, the two cells in Figure 8 were regarded to be in fusion and were recorded as one cell with 147 nuclei. Using this criterion, 8 cells with more than 50 nuclei were regarded to be in fusion.

This study identified cells which formed lacunae on the dentine or pedicel under brush borders as odontoclasts, and 246 odontoclasts including 61 cells in fusion were observed on three specimens. Figure 9 shows the distribution of the number of nuclei per cell of the 246 odontoclasts, from 2 to 147. In the distributions of the number of nuclei per

odontoclast there were not statistically significant differences among the three specimens selected at random ($p > 0.05$). Mononuclear odontoclasts forming a resorptive lacuna were not observed on shedding teeth and there were four cells with more than 100 nuclei. The mean number of nuclei per odontoclast was 21.8 ± 1.2 (SD). A histogram of the number of nuclei per odontoclast showed the distribution to be skewed to the right (toward larger nuclear numbers), and with such a distribution, the mean is strongly influenced by the presence of high values. A more reliable measure of the central tendency is the median, and this was 17, obtained directly from a cumulative frequency curve (Fig. 10). Only 24.4 % of odontoclasts had 10 or fewer nuclei, and 95.5 % had 50 or fewer nuclei. A histogram of the number of nuclei per odontoclast in cell fusion showed that cell fusion occurs independent of increases in the number of nuclei per odontoclast, and that 24.8% of the investigated odontoclasts were in cell fusion (Fig. 9). Table 1 showed a comprehensive comparison of the number of nuclei per odontoclast between Chinook salmon and humans (Domon et al., 1997). In the distribution of the number of nuclei per odontoclast there were statistically significant differences between Chinook salmon and humans ($p < 0.05$).

Discussion

The present study examined the distribution of number of nuclei per odontoclast in Chinook salmon and compared it with that in humans (Table 1), and showed that the range of the number of nuclei per odontoclast in Chinook salmon was larger than that in humans. Regions associated with the replacement of a human deciduous tooth by a permanent one are regions with high tissue turnover, and they are very active physiological resorption sites where a rapid generation of new odontoclasts of high resorptive activity can be expected (Jones and Boyde, 1994). The present and previous studies (Domon et al., 1997) investigated similar resorption sites with high tissue turnover: a teleost shedding tooth and a human deciduous one, however the distributions of nuclei in odontoclasts were significantly different.

The dentition of humans is diphyodont, and the replacement of deciduous teeth occurs only once. The resorption of deciduous teeth by odontoclasts is seen on the surfaces of the root dentine, and this resorption is not continuous but alternates with periods of repair or rest. During periods of rest or repair, the deposition of bone- or cement-like tissue is often observed on the resorbed surfaces of deciduous teeth (Furseth, 1968; Rölling, 1981), and it takes some years till a deciduous tooth is shed in humans. The dentition of Chinook salmon, a teleost fish, is polyphyodont, and the replacement of teeth occurs many times. The situation of tooth replacement in Chinook salmon is quite different from that in humans, and the resorption of shedding teeth by odontoclasts is seen on the inner surfaces of teeth and pedicels, and this resorption is rapid. In rainbow trout, *Oncorhynchus mykiss* (Walbaum), a species related to Chinook salmon, it has been reported that the period for shedding a functional tooth is less than two weeks (Berkovitz, 1977). The present study showed no deposition of bone-like tissue on the resorbed surfaces of teeth associated with replacement. This indicates that

resorption of shedding teeth in Chinook salmon is rapid and continuous and does not alternate with periods of repair or rest.

It is well known that multinucleation of osteoclasts occurs by cell fusion of mononuclear precursors, and that nuclear division does not occur in these cells (Chambers, 1985; Marks and Popoff, 1988; Suda et al., 1992). Therefore, the continuous figures of odontoclasts shown in this study indicate cell fusion. We have observed that cell fusion of odontoclasts was rare on human deciduous teeth (Domon et al., 1998), however the present study showed that 24.8% of the investigated odontoclasts have regarded to be in fusion on shedding teeth of Chinook salmon. These results indicate that cell fusion of odontoclasts in Chinook salmon occurs more frequently than in humans. The present results also showed that mononuclear precursors participated in cell fusion. This means that the generation of new odontoclasts happens on the resorbed surfaces of shedding teeth of Chinook salmon. Summarizing the above, the present results suggest that in shedding teeth of Chinook salmon a rapid generation of new odontoclasts of high resorptive activity induces the increase of the number of nuclei per odontoclast by frequent cell fusion.

In mammals, it is well known that the differentiation of osteoclasts is controlled by complicated signalling pathways; PTH, colony-stimulating factor (CSF), and macrophage colony stimulating factor (M-CSF) (Solari et al., 1996; Amano et al., 1998; Lees and Heersche, 1999). Since PTH is not controlling the osteoclastic differentiation in teleost fish (Huysseune, 2000; Witten et al., 2001). Therefore, the signalling pathways controlling cell fusion of odontoclasts in Chinook salmon might be different from those in mammals, even if there are no findings that are elucidating how the regulation is managed in the teleost osteoclasts or odontoclasts.

It was reported that in advanced teleost fish with acellular bone many mononuclear osteoclasts were seen in bone resorption (Takagi et al., 1989; Witten,

1997; Witten et al., 2001) and in tooth resorption (Van der Heyden et al., 2000). The present results showed no mononuclear odontoclast on shedding teeth of Chinook salmon. The difference of the number of nuclei in osteoclasts and odontoclasts must be considered. First, the methods investigating the specimens are different. Witten (1997), Van der Heyden et al (2000), and Witten et al (2001) showed mononuclear osteoclasts in one section, however we calculated the number of nuclei per odontoclast from the observations of serial 0.5-μm semithin sections. Mononuclear odontoclasts seen on one 0.5-μm semithin section were multinucleated ones when the serial sections of the investigated cells were observed in this study. Witten (1997) and Witten et al. (2001) also used tartrate-resistant acid phosphatase activity (TRAP), the marker enzyme of osteoclasts, and identified TRAP-positive cells as osteoclasts. In their reports, small mononuclear cells which were not regarded as osteoclasts according to morphological criteria showed TRAP-positive reaction. Second, the difference between odontoclasts resorbing teeth and osteoclasts resorbing bone may affect the number of nuclei. The present study examined the number of nuclei in odontoclasts, however Takagi et al. (1989), Witten (1997), and Witten et al. (2001) examined that in osteoclasts. Witten (1997) reported that many TRAP-positive cells containing up to 10 nuclei were seen at the dentary where bone resorption associated with tooth eruption in *Oreochromis niloticus* (Cichlidae). Witten et al (2001) also reported that mononuclear osteoclasts occurred at the neural arches and multinucleated osteoclasts might not be absent from thin skeletal elements in Zebra fish. Therefore, the differences of the number of nuclei may be due to the distinctions of resorption sites and function (e.g. bone surface vs. teeth surface). Third, the differences of the number of nuclei may be due to species differences. Takagi et al. (1989) and Witten (1997) examined osteoclasts in a teleost fish species with acellular bone. The present study examined odontoclasts in a teleost

fish species with cellular bone. Therefore, in teleosts with cellular bone multinucleated odontoclasts may be predominant.

The present study showed a characteristic situation where the inner surfaces of the dental pulp of shedding teeth were filled with numerous odontoclasts. On human deciduous teeth, such a situation has not been observed and the resorbed surfaces are not filled with odontoclasts (Domon et al., 1994). It is reported that mammalian osteoclasts alternate resorption with migration and this alternation makes the resorbed surfaces smooth (Jones et al., 1984). The present study showed that odontoclasts were situated close to one another on the resorbed surfaces in Chinook salmon. This finding indicates that odontoclasts in Chinook salmon continue to resorb and hardly alternate resorption with migration. It appears that such one-sided resorption does not make the resorbed surfaces smooth but makes the margins among lacunae sharp. However, in the present results most of the outlines of the resorbed surfaces were smooth although the contours of the lacunae were wavy and some margins among lacunae were sharp. The present study showed that cell fusion occurred on adjacent odontoclasts independent of increases in the number of nuclei per cell. Odontoclasts after fusion probably resorb such sharpened margins among lacunae on the resorbed surfaces. Therefore, it is postulated that to make the resorbed surfaces smooth (to make the margins among lacunae smooth) the multinucleation by cell fusion frequently occurs on adjacent odontoclasts in Chinook salmon independent of the number of nuclei. This may be a reason for the multinucleation in odontoclasts of Chinook salmon, and this multinucleation may occur to mammalian osteoclasts. Multinucleation is a characteristic of osteoclasts and/or odontoclasts, however its reason remains unknown. Further studies of the distribution of nuclei in other non-mammalian vertebrates will be needed to elucidate this.

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Legends

Fig. 1. Light micrograph showing a shedding tooth of the mandible. The inner surfaces of the tooth (T) are filled with numerous odontoclasts (arrows), and odontoclasts are situated close to each other from the pedicel (PE) areas to the top of the pulp cavity (PC). Most of the outline of the resorbed surfaces is flat, but there are sharpened margins among the lacunae. The boxed area indicates the site of the magnification in Figure 2. x 100. Bar = 100 μm.

Fig. 2. Light micrograph showing odontoclasts in the boxed area in Figure 1. Odontoclasts (arrows) formed resorptive lacunae (asterisks) under the brush border (BB) on the dentine surfaces (D). PC: pulp cavity. x 350. Bar = 50 μm.

Fig. 3. Electron micrograph showing the ruffled border of an odontoclast. The ruffled border (RB) is seen against the dentine surface (D). Under the ruffled border, the collagen fibrils (arrowheads) composing the dentine are disrupted. x 9,200. Bar = 1 μm.

Fig. 4. Serial semithin sections of an odontoclast, a-h respectively 5.0, 15.5, 21.5, 26.5, 33.5, 41.5, 50.5, and 56.5 μm from the cell surface. The nuclei are numbered in the order they appear on the sections. The serial sections show that this cell has 18 nuclei (numbered arrows). This cell forms a resorptive lacuna (asterisks) on the dentine (D) under the brush border (BB). The cell is not in contact with other cells. Large arrows (a, h) indicate the edges of the cell. x 700. Bar = 15 μm.

Fig. 5. Model showing the three-dimensional reconstruction of the 18-nuclear odontoclast in Figure 4. This image shows the dentine (yellow), cellular surfaces (blue), and nuclei (red). The odontoclast shows a rounded outline on the dentine. The cell surface

is displayed as semitransparent, and the 18 nuclei are scattered in the cytoplasm. This image is displayed looking obliquely to the resorbed surface. x 780. Bar = 15 μm.

Fig. 6. Serial semithin sections of two odontoclasts (13-nuclear and 17-nuclear ones) sectioned in the perpendicular direction to resorbed surfaces, a-h respectively 1.5, 6.5, 11.0, 15.0, 20.5, 26.5, 30.0, and 33.0 μm from the cell surface of a 13-nuclear odontoclasts. The nuclei are numbered in the order they appear on the sections. The sections show that one odontoclast has 13 nuclei in the cytoplasm (a-e), and the other has 17 nuclei (c-h). These odontoclasts form resorptive lacunae (asterisks) on the dentine (D) under the brush border (BB). The cellular continuity between two cells is observed in 6e (arrowhead). Two arrows in 6a indicate the edges of the 13 nuclei odontoclast, and an arrow in 6h indicates the edge of the 17 nuclear odontoclaststst. The odontoclasts are situated close to one another on the dentine. x 740. Bar = 15 μm.

Fig. 7. Model showing the three-dimensional reconstruction of the 13-nuclear and the 17-nuclear odontoclasts in Figure 6. This image consists of the dentine (yellow) and cellular surfaces (blue), and nuclei (red). The two odontoclasts show rounded outlines on the dentine, and are in contact at a narrow bridge (arrowhead). The 13 and the 17 nuclei are scattered in the cytoplasm of each cell. This image is displayed looking obliquely to the resorbed surface. x 900. Bar = 15 μm.

Fig. 8. Serial semithin sections of two odontoclasts (94 and 53 nuclear ones), a-h respectively 8.5, 20.5, 30.0, 39.5, 48.0, 57.5, 66.5, and 71.0 μm from the cell surface of a 94-nuclear odontoclasts. The nuclei are numbered in the order they appear on the sections. The sections show that one odontoclast has 94 nuclei in the cytoplasm (a-f), and the other has 53 nuclei (d-h). These odontoclasts form resorptive lacunae (asterisks) on the dentine

(D) under the brush border (BB). The continuity between the two cells is observed in 10e (arrowhead). x 265. Bar = 50 μm.

Fig. 9. Histograms showing the number of nuclei/cell in 246 odontoclasts (shaded columns) and 61 odontoclasts in cell fusion (white columns). The 246 cells include 61 cells in fusion. This histogram shows that cell fusion increases with increases in the number of nuclei/cell.

Fig. 10. Cumulative frequencies of Chinool salmon odontoclasts (white columns) and human odontoclasts (black columns; Domon et al. Anat Rec 1997; 249: 449-457) with different numbers of nuclei.

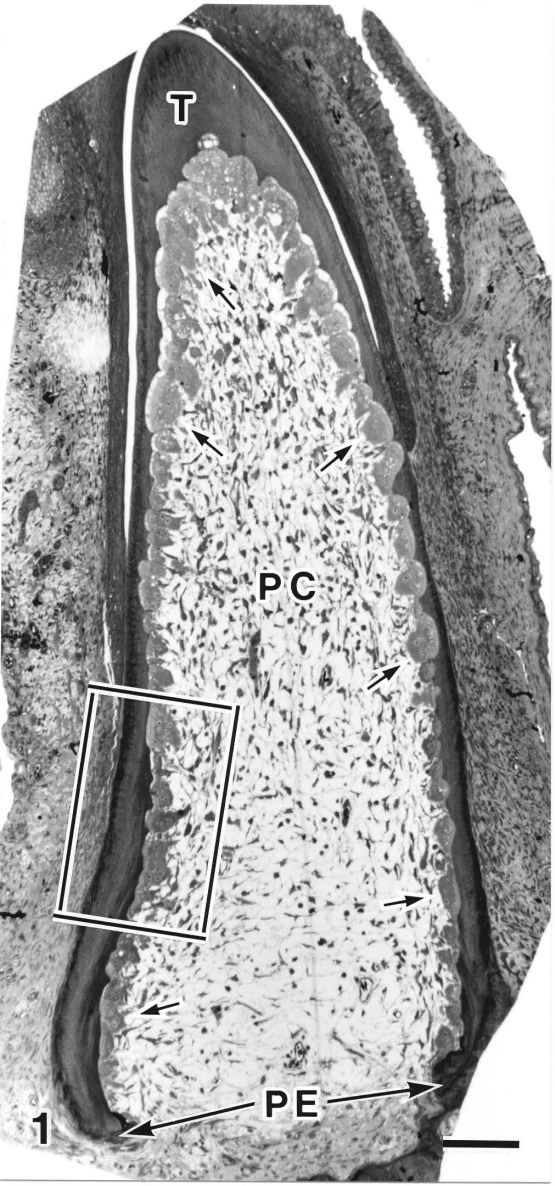
Table 1. Number of nuclei per odontoclast

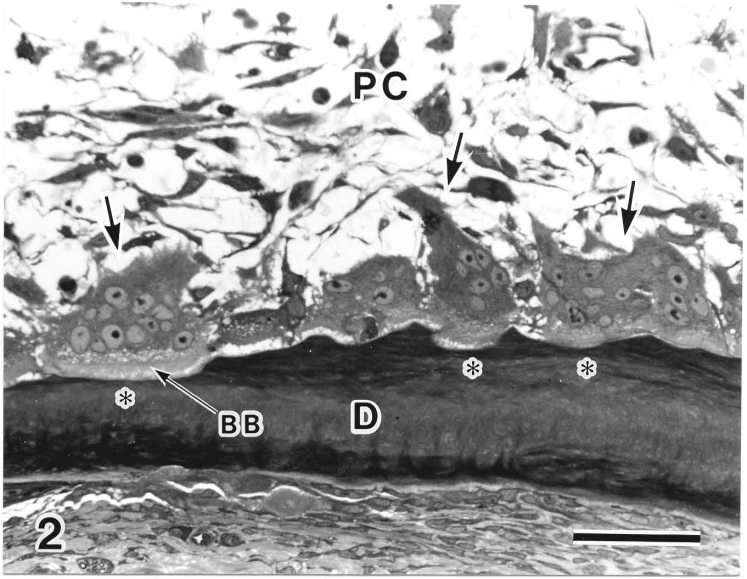
Species	n	Mean	SD	Median	Max. nuclei	Relative frequencies of cells with 10 or fewer nuclei (%)
Human*	243	5.3	3.2	4	28	93.8
Chinook salmon	246	21.8	1.2	17	147	24.4

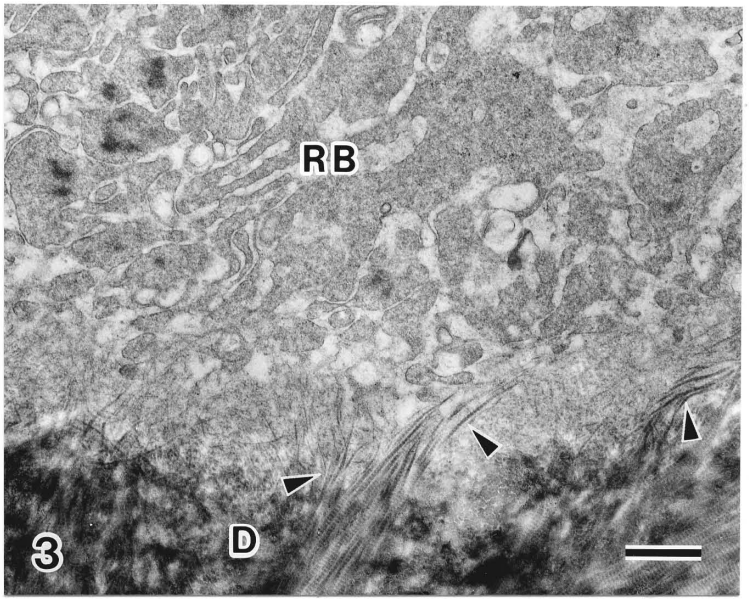
* Domon et al. (1997) Anat Rec 249: 449-457.

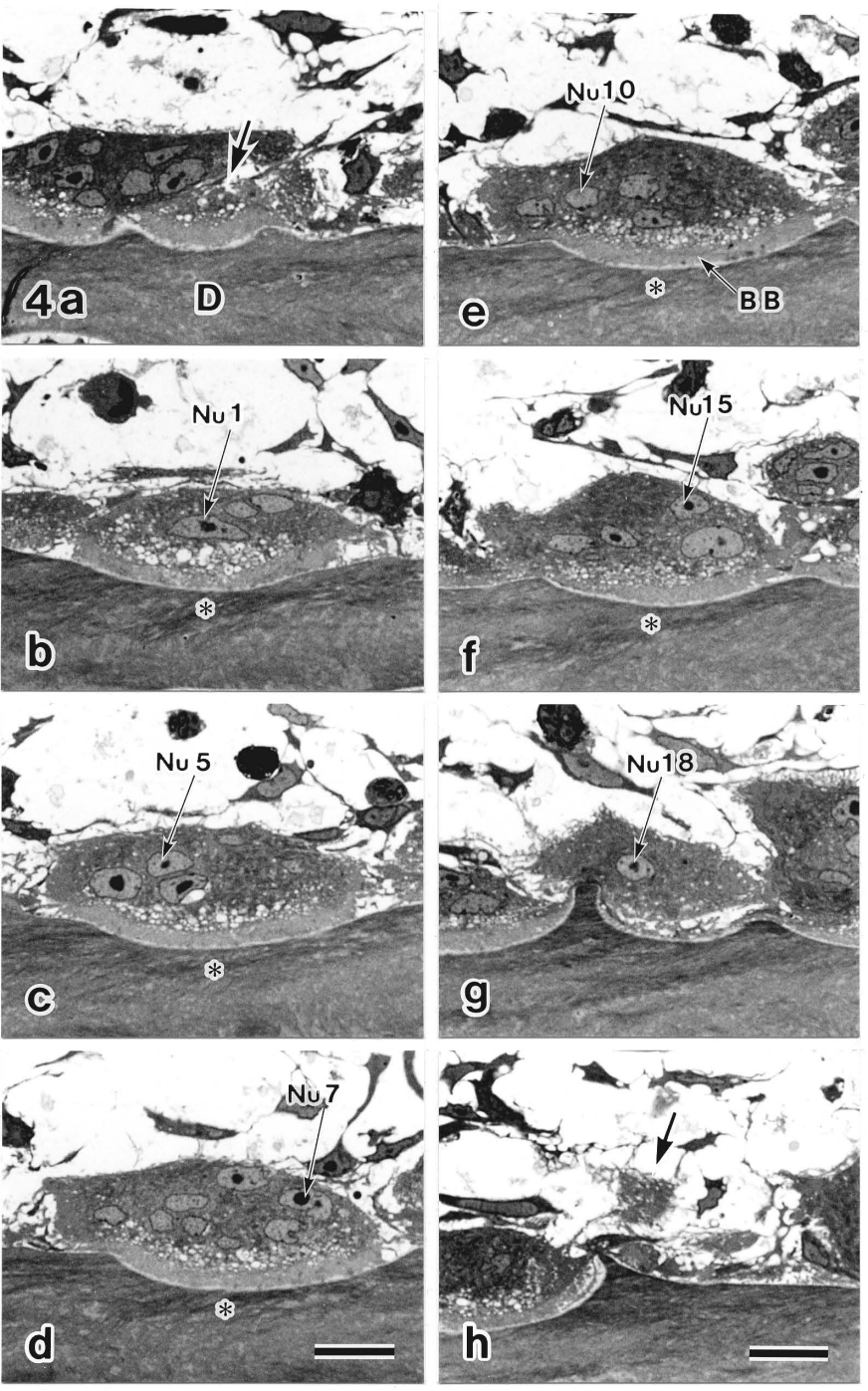
Table 1

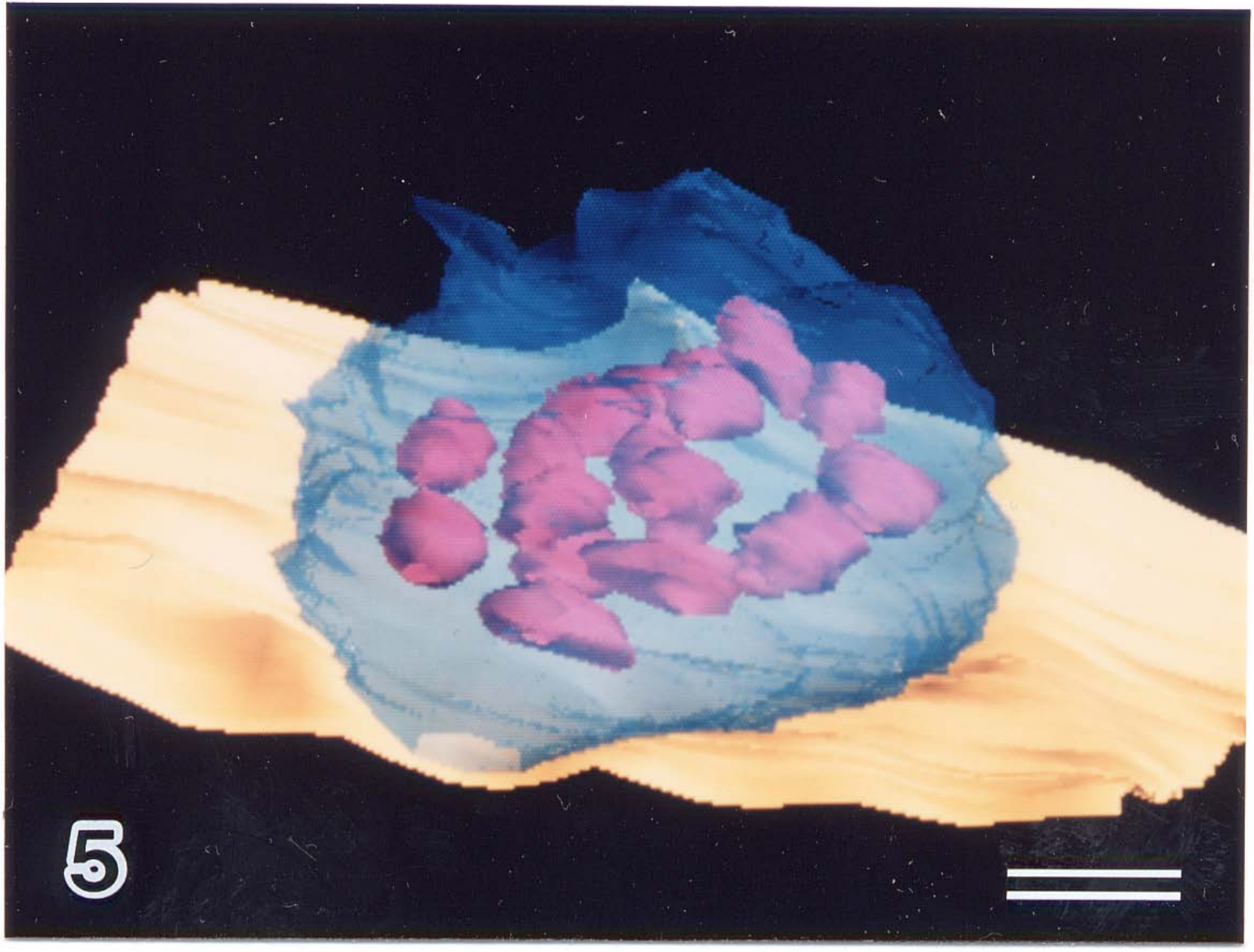
Domon et al.

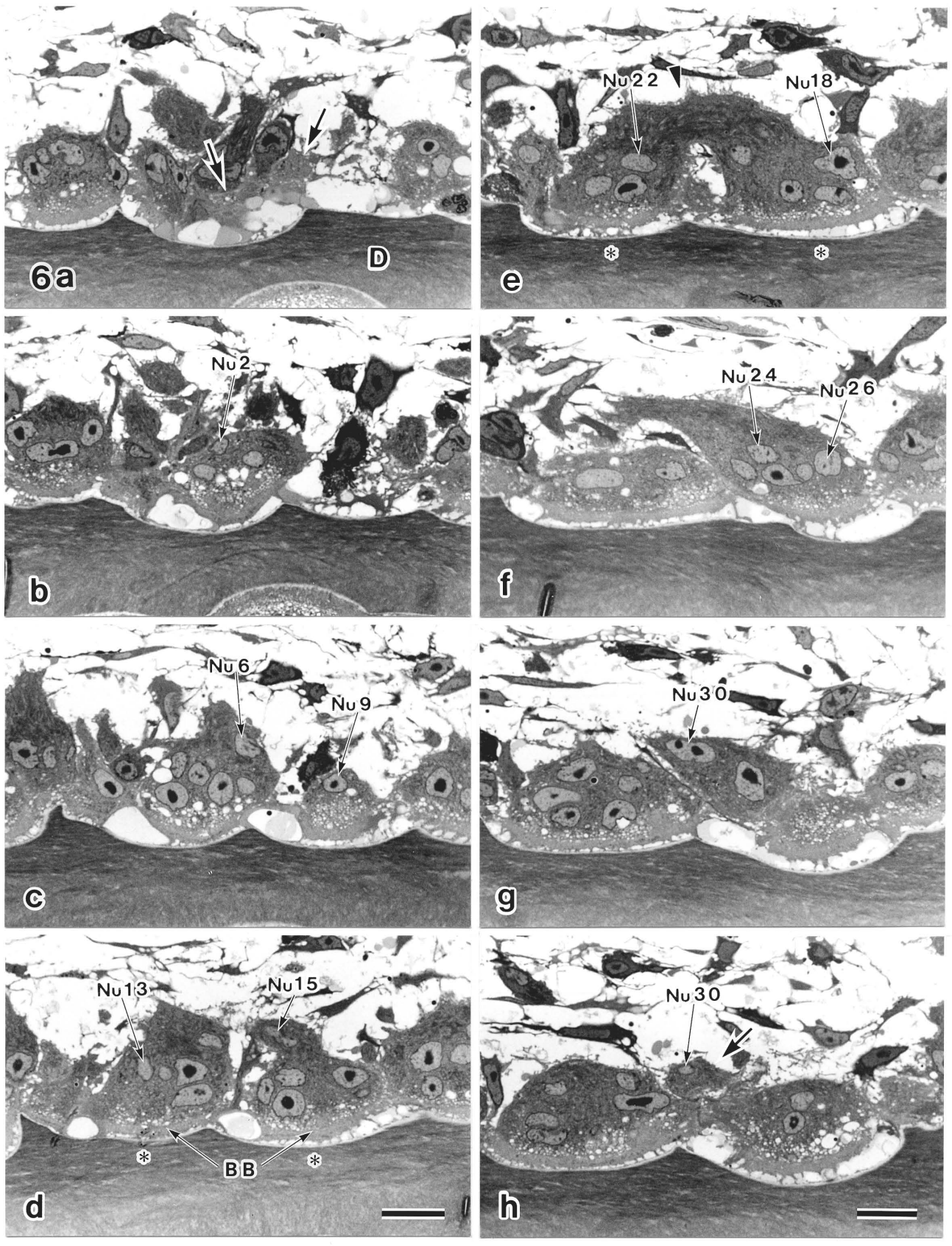

T
PC
PE
1

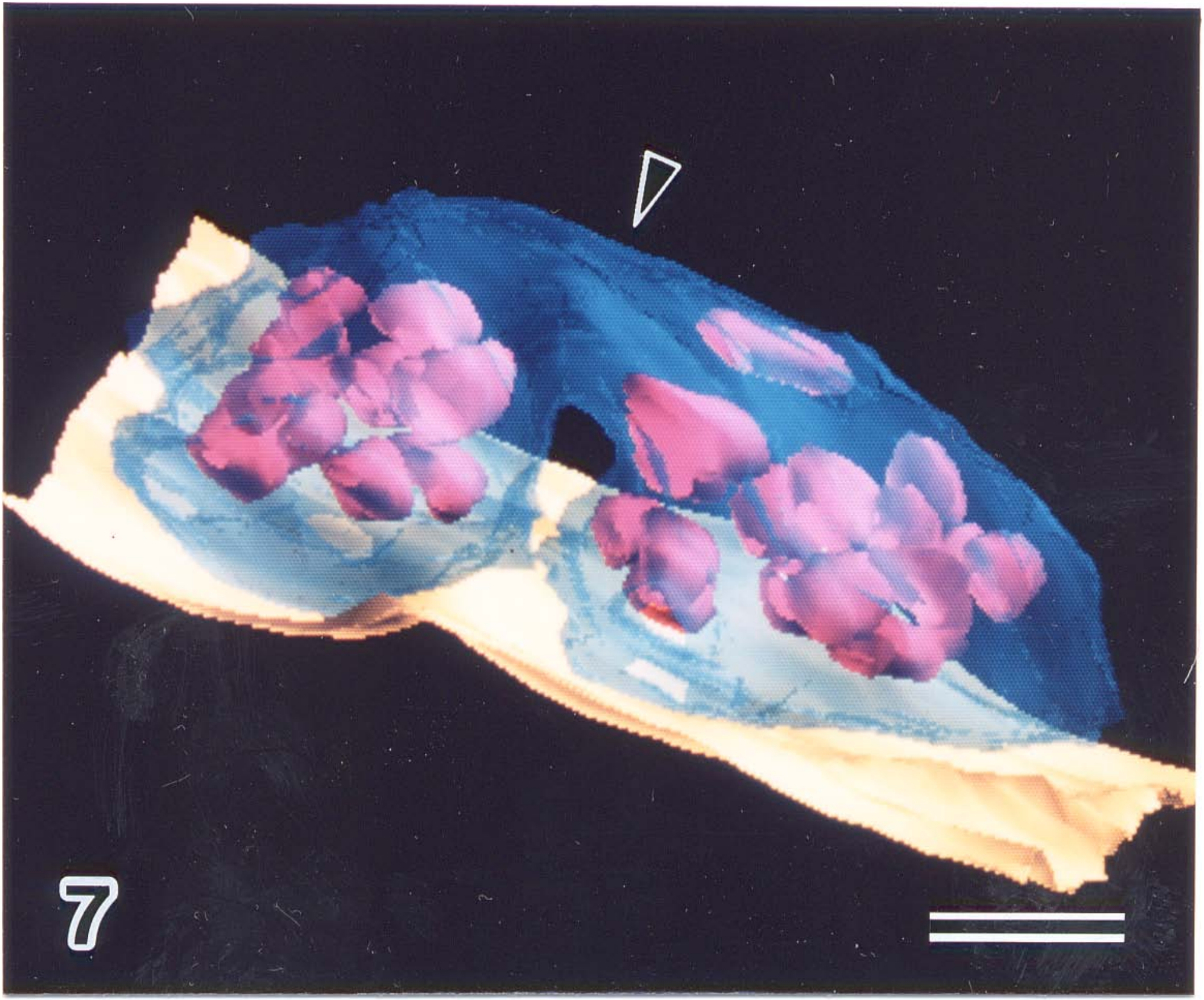

PC
BB
D
2

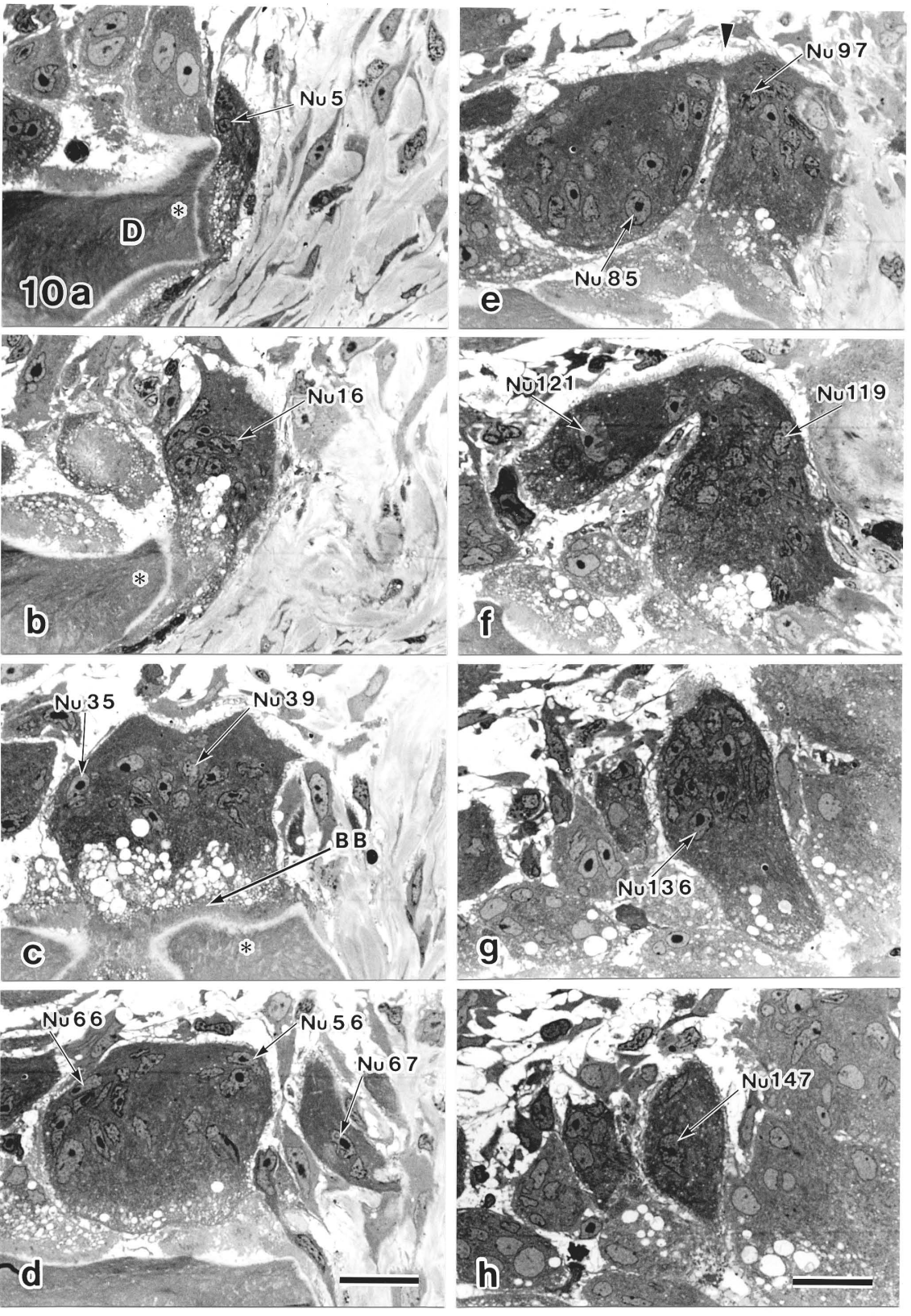

RB
D
3


4a
D
Nu10
BB
e
Nu 1
b
Nu15
f
Nu 5
c
Nu18
g
Nu 7
d
h


5


6a
D
e
Nu22
Nu18
b
Nu2
f
Nu24
Nu26
c
Nu6
Nu9
g
Nu30
d
Nu13
Nu15
BB
h
Nu30


7


Nu 5
D
10a
Nu 97
Nu 85
e
Nu16
b
Nu121
Nu119
f
Nu 35
Nu 39
BB
c
Nu136
g
Nu 66
Nu 56
Nu 67
d
Nu147
h

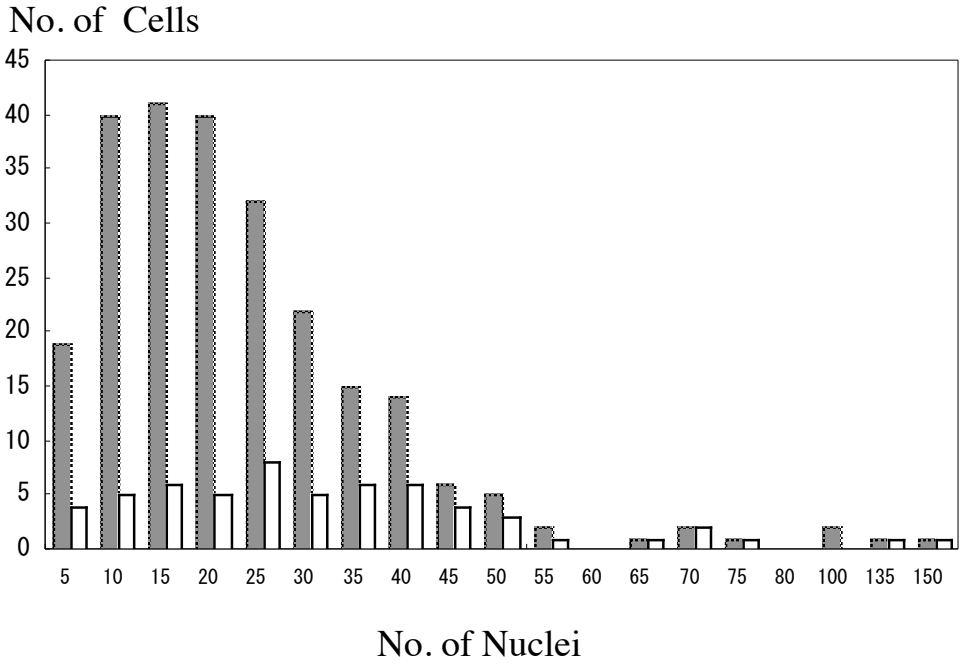


Fig. 9
Domon et al.

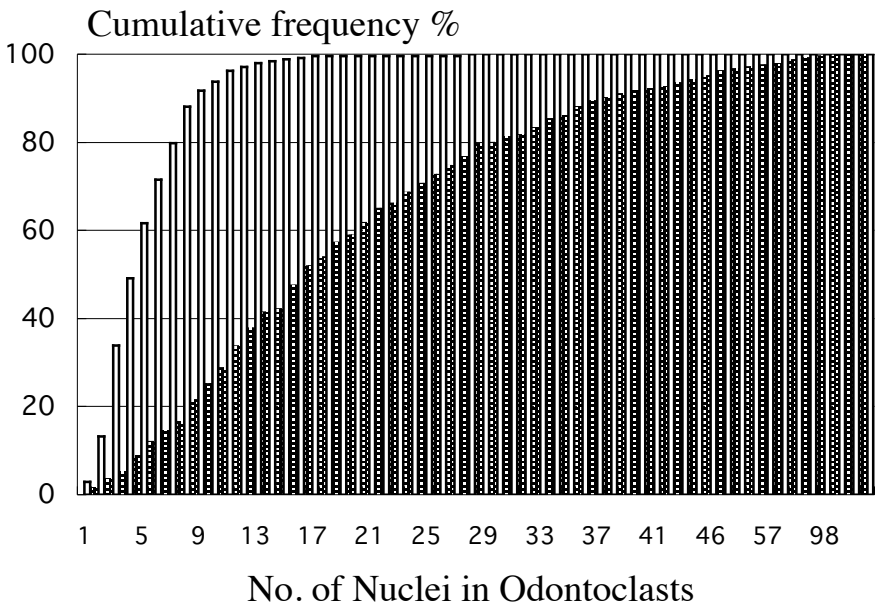


Fig. 10
Domon et al.