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Authors: Rufina S.Y. Cheng, M.S.M. Wai, Gigi C.T. Leung, Tony C.H. Chow, W.W. Sze-To, D.T. Yew

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Different types of visual cells in the photoreceptor layer of the retinae of the treeshrew (Tupaia belangeri chinensis) as revealed by scanning microscopy

Rufina S.Y. Cheng et al., Photoreceptor layer of retinae of the treeshrew

Rufina S.Y. Cheng[1], M.S.M. Wai[2], Gigi C.T. Leung[1, 3], Tony C.H. Chow[1, 3], W.W. Sze-To[1], D.T. Yew[1, 2, 3]

[1]School of Chinese Medicine, The Chinese University of Hong Kong, Shatin, Hong Kong

[2]School of Biomedical Science, The Chinese University of Hong Kong, Shatin, Hong Kong

[3]Hong Kong College of Technology, Shatin, Hong Kong

Address for correspondence: D. T. Yew, Room 101, Li Wai Chun Building, Chung Chi College, The Chinese University of Hong Kong, Hong Kong; tel: (852) 39434140, e-mail: david-yew@cuhk.edu.hk

ABSTRACT

The retinae of treeshrew have never been evaluated by scanning electron microscopic studies. This work described the visual cells in the photoreceptor layer of the retinae of treeshrew (Tupaia belangeri chinensis) living on the high plateau of Yunnan, China, via scanning electron microscopy. Results indicated five different types of cones morphologically, in which two of those have shown oil droplet like structures in their inner segments. To our knowledge, no prior studies have reported oil droplets in the visual cells of higher mammals, only in lower vertebrate and primitive mammals. In addition, this study revealed one type of degenerative visual cell without outer segments. The findings signal the needs for additional studies to understand the

physiological functions and phylogenetic relationships of the diversity of visual cells in this group of mammal.

Key words: Retina, Tupaia belangeri, treeshrew, cones, rods, scanning electron microscopy

INTRODUCTION

Treeshrews are small mammals found widely across Southeast Asia, including Malaysia, Singapore, Thailand, Indonesia, and the Yunnan Province of China. The animal was first discovered in 1820 by two French zoologists, Diad and Duvancel. To date, scientists have classified the animal into two families (Tupaiinae and Ptilocercinae) under the order Scandentia. The genus *Tupaia* is the largest group of all and contains the highest number of species found. Two common species known in this group are *Tupaia glis* and *Tupaia belangeri*. Most of the discovered *Tupaia* treeshrews are diurnal animals with only the *Ptilocercus* species being nocturnal. Some treeshrews are arboreal (1), and they usually live on small trees that allow them to leap from one tree to another. Treeshrews, being omnivorous (2), obtain nutrients from juicy fruits, seeds, and insects. They have an average life-span of 12 years, just like pet dogs.

Treeshrews were initially classified as insectivores pre-1966, but they were later grouped under the order of primate, then under scandentia (3). Hasler and Sorenson (1974) conducted a behavioral study on *Tupaia chinensis* and reported high activities of these animals are found between 07:00 and 17:00 of the day (2). They spend most of their time on trees and move around by hopping. Tate (1947) reported these animals usually stayed at altitudes of 1500-8000 ft., while spending half of their time searching for food in summer (4). In general, treeshrews rely on their vision more than olfaction (2).

The eyes of the treeshrews have been studied for almost half a century. The retinae of the *Tupaia* species have more cones with about 4-5% rods (5), while the *Ptilocercus spp.*, being nocturnal animals, might have retinae consisted mainly of rods. Either way, documented by a study performing funduscopy on *Tupaia glis* (3), no macula was found in the retinae of these animals.

Müller and Peichl (1993) studied the retinae in whole mount and semi-thin sections , they later concluded that the cones in the treeshrew retinae were two to ten times the numbers of rods in the retinae (6). Electron microscopic studies on the visual cells demonstrated that the cone cells had large and round groups of mitochondria at the apex of the inner segments (i.e. the apex of the ellipsoid region), which was termed as the "lens mitochondria" (7). In fact, large number of mitochondria spread all the way downwards to the lower regions of the inner segments of cones, where they formed again dilated regions at the bases of these inner segments (7). Recently, the treeshrews have been introduced as a model for myopic research (8).

Looking at other parts of the visual system in treeshrews, Campbell (1966) reported cross and uncross nerve fibers in the optic nerve of the *Tupaia spp. (9)*. In the lateral geniculate bodies, lamination was demonstrated in *Tupaia spp.* (9), while no lamination was observed in the nocturnal species of *Ptilocercus spp.* (10).

Despite the above findings, the different types of visual cells with different three-dimensional morphologies in the retinae of the treeshrews had never been recorded in the literature. Thus, we report here the different morphologies of various types of visual cells in the photoreceptor layer of the retinae of *Tupaia chinensis* observed under scanning electron microscopy.

MATERIAL AND METHODS

Animals and treatment

Five young adult treeshrews of the species *Tupaia chinensis* of an average of 4 inches in length (not including tails) were reared in the animal house of Yunnan Medical University. Unfortunately, these animals did not survive in long captivity and the brains and eyes were given to us in fixation upon death of the animals. The study was approved by the Research Coordination and Development Committee of the Hong Kong College of Technology, Shatin, New Territories, Hong Kong.

Tissue preparation for scanning electron microscopy

The retinae were fixed in 2.5% glutaraldehyde buffered at pH 7.4 in 0.1M Sorensen's phosphate solution for one day. The eyes were bisected into anterior and posterior halves. The posterior halves with retinae were then washed in 0.1M Sorensen's buffer at pH 7.4 for three times, 10 mins each. 1% aqueous osmium tetroxide was used to post-fix the tissues for 2 hrs. The tissues were rinsed again with 0.1M Sorensen's buffer (pH 7.4) for three times, 10 mins each. Afterwards, the tissues were dehydrated in graded ethanol for 5 mins of different concentration. Drying of the tissues was done with a Ladd critical point dryer (40^0C, 1300psi), using liquid CO_2 as the drying agent, and followed by cooling. The retinal layer with the pigment epithelium were mounted on a copper stub with double-sided sticky tape, followed by gold palladium coating in a sputter coater (Edwards S150B Sputter Coater). The retinae were ready for scanning under electron microscopic examination (JEOL JSM 6301F, Japan).

For semi-morphometry, the retinae of three animals were divided in to three equal sections, namely the central, the midperiphery, and the periphery regions. The central region was adjacent and nearest to the optic nerve as the retina had no macula. The periphery region was adjacent to the limbus. The region in between the central and periphery region was termed as midperiphery. After treatment of the retinae for scanning microscopy, three random areas of size 100 μm^2 were taken from each region of each animal; and the numbers of rod cells observed under the scanning electron microscopy were expressed as a percentage of the numbers of total photoreceptors in those areas for each region. Statistical analysis was conducted using Prism 6 (GraphPad Software, USA). Comparison of means was performed using one -way ANOVA and post-hoc Tukey's Test. P-Values of <0.05 were considered statistically significant.

RESULTS

General remarks

General morphological survey: The retinae of the treeshrews had closely aligned visual cells. Scanning electron microscopy indicated clusters of retinal pigment epithelial cells as well as clusters of phagosomes in the ventricular space amongst the visual cells of the photoreceptor layer (Fig. 1). There were more cone cells found in the central region of the retinae than rod cells (Fig. 2), with some of the cones tightly packed together (Fig. 2). From the midperiphery onwards to the periphery region, increasing rod cells was observed. Rods were not found to be in small quantity in the species of *Tupaia chinensis* studied by us.

Specific visual cells

The *Tupaia* treeshrews' retinae have slender and elongated rods with the outer and inner segments as roughly the same diameter under the coronal view (Fig. 3). The outer segment of the rods were of 1 µm - 1.5 µm in diameter and the inner segments were about 1 µm in diameter. In the dark-adapted retinae, the longest outer segment observed in rod cells was more than 10 µm. Sometimes, the tips of the outer segments of rods could be inclined at an angle (Fig. 3).

There were at least five morphologically different cone cells found in the treeshrews. The first type of cones (cone A) (Fig. 4a) had an inner segment of 4 µm – 5 µm in length; with a long ciliary stalk, reaching the length of 1 µm - 2 µm, and attaching to the outer segment. In rare cases, the ciliary stalk looked bifurcated (Fig. 4a). The outer segments of cone A were blade-shaped structures with average of 2.5 µm - 4 µm in length and diameter up to 1 µm (Fig. 4a).

The second type of cones (cone B) were of two types (B1 and B2). B2 outer segments are generally conical in shape and have short inner segments at an average of less than 4 µm in height with a short 0.5µm diameter (Fig. 4a). Cone B1 was more slender (B1) (Fig. 4a) and had a relatively dilated distal tip (the ellipsoid) of the inner segments (Figs. 4a, 4c), while some B1 cones had oil droplets (Fig. 4b). All B1 cones had no significant cilia (Figs. 4a, 4b, 4c). The ellipsoids housing the oil droplets contained the lens' mitochondria below as described in the literature (11). Cones B1 and B2 in many retinae were often seen in close proximity.

The third type of cones (cone C) has a long and strong cilium of 1.5 μm - 2 μm long in coronal view, extending from the 4 μm tall inner segment (Fig. 4e). Very few cone Cs have distally expanded inner segments (Fig. 4c).

Cone Ds had round bodies of inner segments averaged 4 μm in length with curved outer segments of 4 μm in length that appears to be continuous (Fig. 4d). The outer segment, when magnified, appeared as a cutting curved blade of a farmer's knife (Fig. 4d). The base of the blade, at average, was about 2.5 μm in diameter with a tip of about 0.25 μm.

Apart from the forementioned types of cones, there was another type of cone with a broad and large proximal base of inner segment, which we named as cone E (Fig. 4e). These cones also had globular oil droplets (Fig. 4e).

Occasionally, long slender inner segments ending with cilia were also observed. As there was no obvious outer segment seen in this type of cone cell, we named it as immature or degenerative cones F (Fig. 4f). Although labeled as a cone, it was also possible that it was a degenerating rod. Shedding of the tips of normal rods and cones at the outer segments was observed, resulting in the globular phagosomes in both cases (Fig. 5a, 5b).

Our morphometric studies revealed fewer number of rod cells found in the central region than the midperiphery and the periphery regions. The amount of rod cells observed were, however, not as few as those reported earlier (5, 6, 12) ($p < 0.05$, Figure 6). A lot of the previous studies conducted their observations with transmission electron microscopy or with histology, thus the lack of 3D morphology hampered the identification of visual cell types.

DISCUSSION

Samorajski et al. (1966) studied retinae of *Tupaia glis*, using electron microscopy and histology (3). The team reported the absence of macula in *Tupaia glis*, and the presence of both large and small sized cones together with rods in the retinae, with the latter at the periphery. Van Dongen et al. (1976) also reported a higher proportion of cone cells among visual cells observed in the retinae of treeshrews (13). Kuhne (1983) concluded there were only 5 rod cells in the retinae among hundreds of cone cells (12). Later, Immel and Fisher (1985) reported 5% of rod cells in the retinae (5). In our study, more rods are demonstrated compared to those previous findings in the literature, though the rods were mostly in the periphery, they were not so few in number (13).

We unveiled the various types of morphologically diversified cones in the treeshrews' retinae. Mosaic of cones were recognized in these animals by modern technique using optical coherence tomography (14). Cone A were found to dominate the central retinae where rods were rarely seen. This cone type contains a blade-shaped outer segment, which connects to the inner segment with a long cilium. Cone B was with the classical conical-shaped outer segments, which appeared to coincide in shape with the red spectrum cones suggested by Müller and Peichl (1989) in their transmission electron microscopy study (11). Two types of B cones were discovered, B2 had short cilia while B1 had no visible cilia between the outer and inner segments. B1 contained an oil droplet inside the ellipsoid of the inner segment. The other two types of cones (cone C and E) were different in shape, with cone C being slender and with a long cilium, while cone E had a broad-based inner segment. As cone A were found to be abundant in the treeshrews studied here and considering treeshrews being active under the sun in high plateau areas, these cone As may be related to the yellow spectrum of vision. The other three types of cones, B, C and E, were probably related to the two remaining light spectra of blue and green wavelengths documented in the literature (7, 11). The oil droplet found in cone E and B1 might act as filters for certain wavelengths similar to those found in chickens (15). Since the blue spectrum is near to the ultraviolet spectrum, it is likely that those cones are located in the peripheral retina. Cone D, with the presence of hook-like outer segments, had never been reported in vertebrates. Finally, cone F visual cells had a cilium and no outer segment, and were speculated to be vestigial or degenerating structures. Developmental defects in the retinae have been documented in cave animals and nocturnal animals, such as fish and serpent snake (16-18), or in degenerative cones that have lost their outer segments (19). Considering the extrusion of cell bodies into the interreceptor matrix in the eyes of these animals (unpublished histological observation) and that the habitat of these animals is on the highland exposed to bright sunlight and ultraviolet radiation, some retinal degeneration is possible. Intense light causing retinal degeneration in addition to lens degeneration had been documented in the rodents (20). One of the moves treeshrews took to protect themselves from light damage is the choice to live on short trees and branches rather than on the top of tall trees. The treeshrews, once were termed as a primitive “primate”, deserved to be investigated further for its evolutionary and biological values.

The various cone cells reported in treeshrews in this study are similar to those found in lower vertebrates, e.g. fish, turtle (21, 22), birds (15, 23) and in primitive mammals like platypus (24) but not in monotremal anteaters (25), in that some of their cones displayed oil droplets. Oil droplets could act as microlens for spectral filtering (26). As far as these were concern, retinae in treeshrews retained primitive features not found in most mammals.

CONCLUSIONS

In here, we were able to distinguish various novel types of cone cells by Scanning Electron Microscopy in the photoreceptor layer of the treeshrews’ retinae. The differences in physiology of each cone cell type should be taken into further studies and hopefully the phylogenetic position of the treeshrew could also be further evaluated.

Conflict of interest: None declared

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Figure 1. Central retinae of treeshrew. P denotes phagosome and B denotes a cluster of pigments. Scale bar is 10 µm.

Figure 2. Central areas of the retinae displayed many different cones (C1 – C5). C2 was shorter in length. Rods (R) were few. Scale bar is 10 µm.

Figure 3. Treeshrews' retinae indicating a rod (R), rod outer segments (Ro), a rod inner segment (Ri). B denotes a type B1 cone and B0 denotes its conical outer segment. Very short cilium (if any) was seen between outer and inner segment of this type of cone. Scale bar is 1 µm.

Figure 4. A. Cone types A and B. In cone A, Ao indicates blade-like outer segments, Ai denotes an inner segments and Ac denotes a cilium. An arrow shows possible

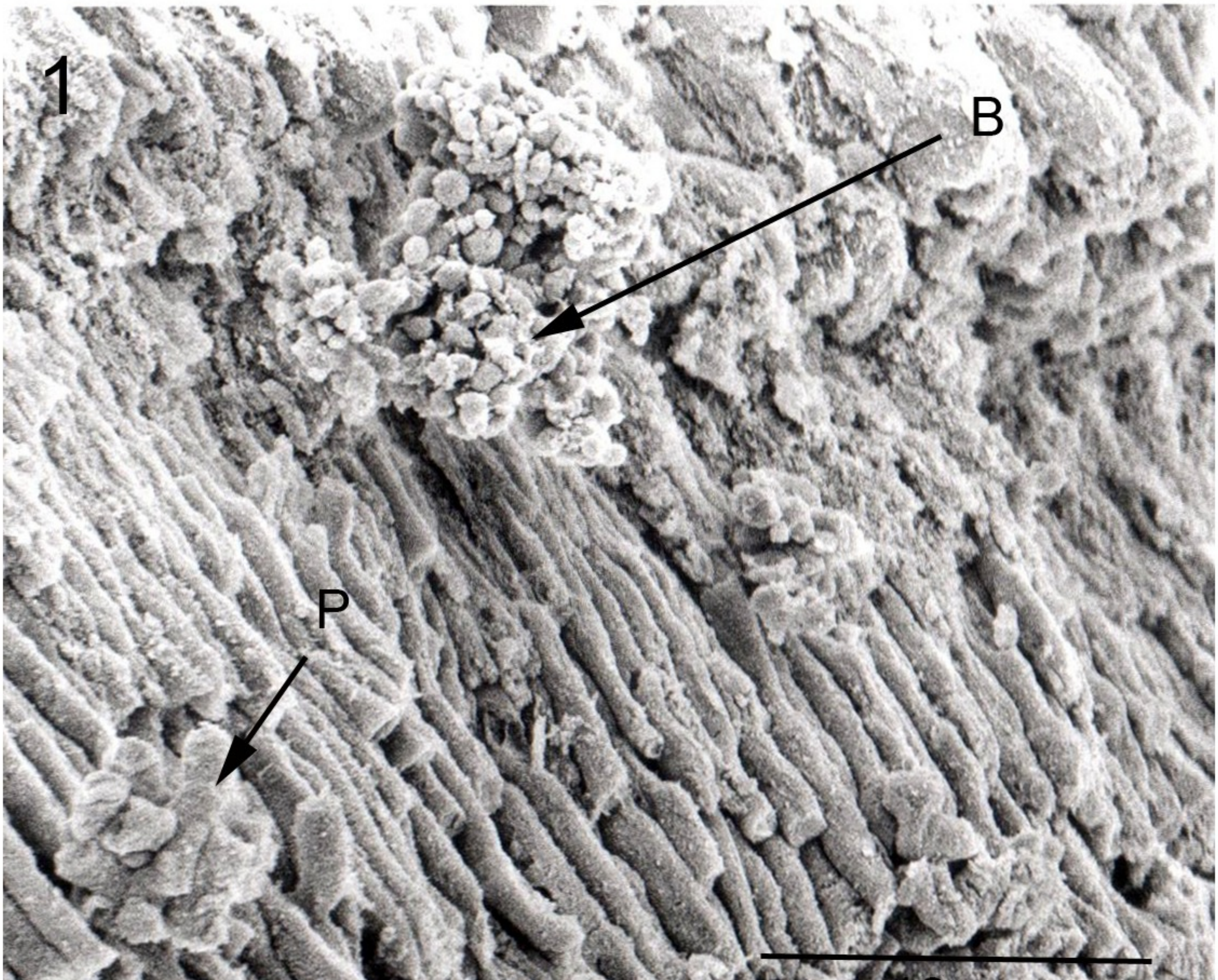
bifurcation. Bo denotes outer segment tip of a B1 cone, and Be indicates ellipsoid region of the cone B1. Bi indicates thick inner segment of a cone B2. There were two types of B cones, namely B1 and B2. B1 contains unapparent cilia between outer and inner segments while B2 contains very short cilia. Scale bar 1 µm; **B.** Arrows indicate oil droplets in cone B. Scale bar 1 µm; **C.** Treeshrews' retinae showing an inner segment (Ci) and an outer segment (Co) of cone C. Cone Cs were usually longer and slender with a long cilium (CI). B1 indicates B1 cones with large ellipsoid in inner segment. Scale bar 1 µm; **D.** B2 is a small cone with a short cilium (Bc). Bi denotes the inner segment and Bo indicates conical outer segment. D is a large hook-like outer segment of cone D, which contains a bulging and short inner segment. Scale bar 1 µm; **E.** A cone E with dilated inner segment at base and an oil droplet at top of inner segment (arrow). Scale bar 1 µm; **F.** A possibly degenerating cone F (F) with straight or hook like cilium (C) and no outer segment. Scale bar 1 µm.

Figure 5. Outer segment shedding in rod A (**A**) and cone B (**B**). When plasma membrane ruptures, globular phagosomes (arrows) are generated. Scale bar 1 µm.

Figure 6. Relative proportion of rods (in density) in the three regions of the retinae; **** denotes $p \leq 0.0001$.

Table of measurement (ANOVA). Total percentage of rod cells in respective areas of the retina

ANOVA table	**SS**	**DF**	**MS**	**F (DFn, DFd)**	**P value**
Treatment (between columns)	1.540	2	0.7701	F (2, 24) = 209.2	P < 0.0001
Residual (within columns)	0.08835	24	0.003681		
Total	1.629	26			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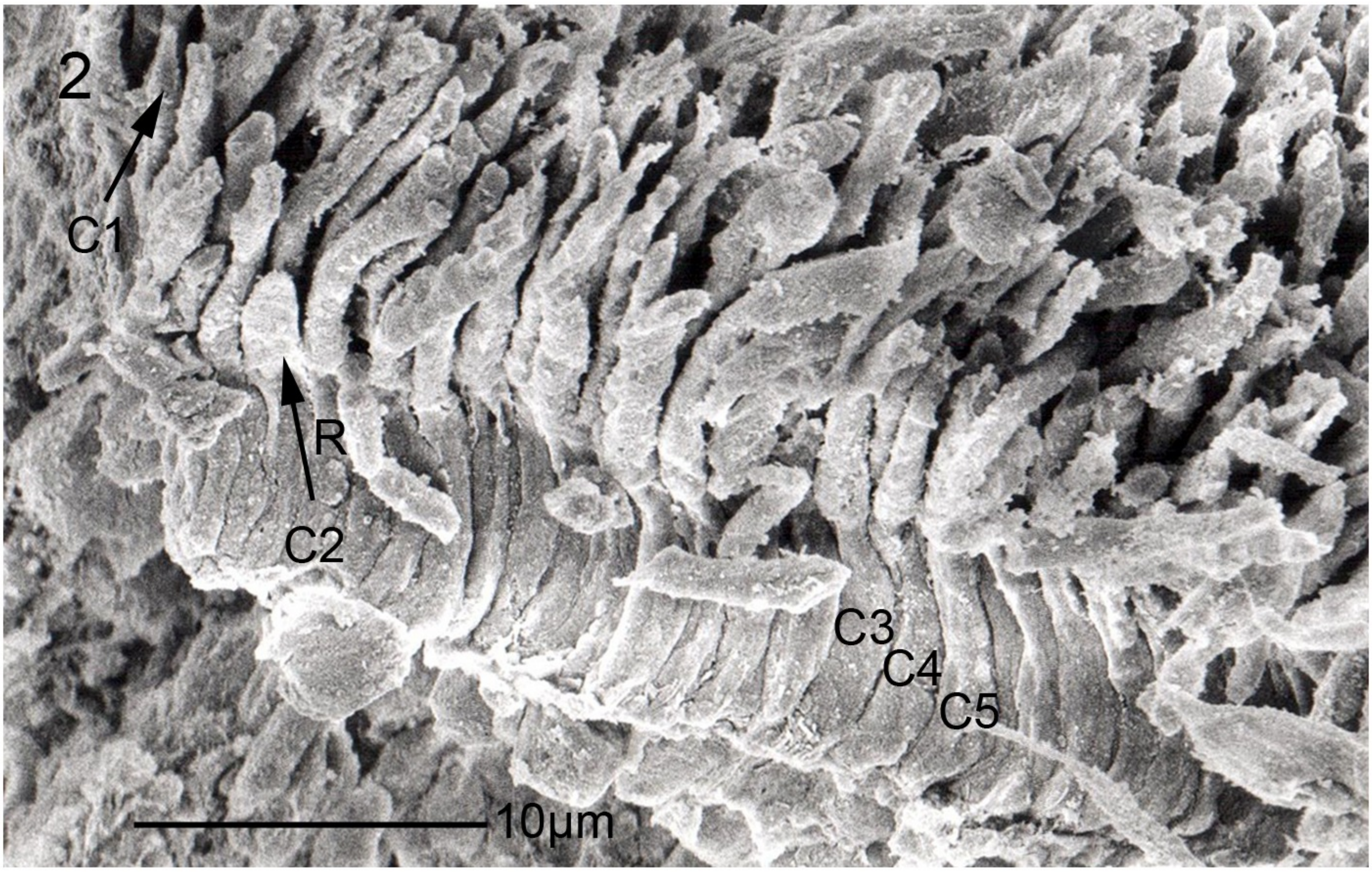
B
P
10µm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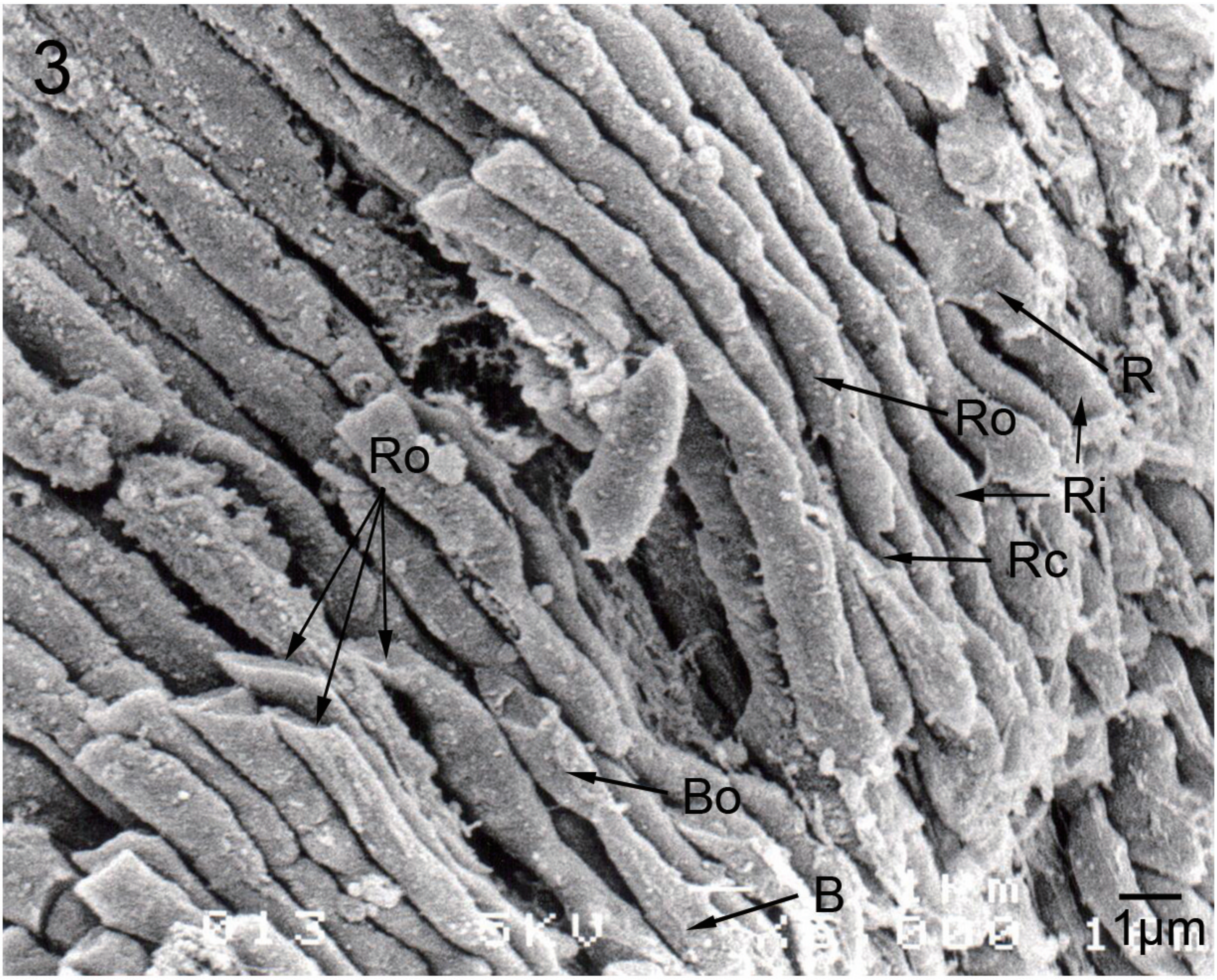
C1
R
C2
C3
C4
C5
10µm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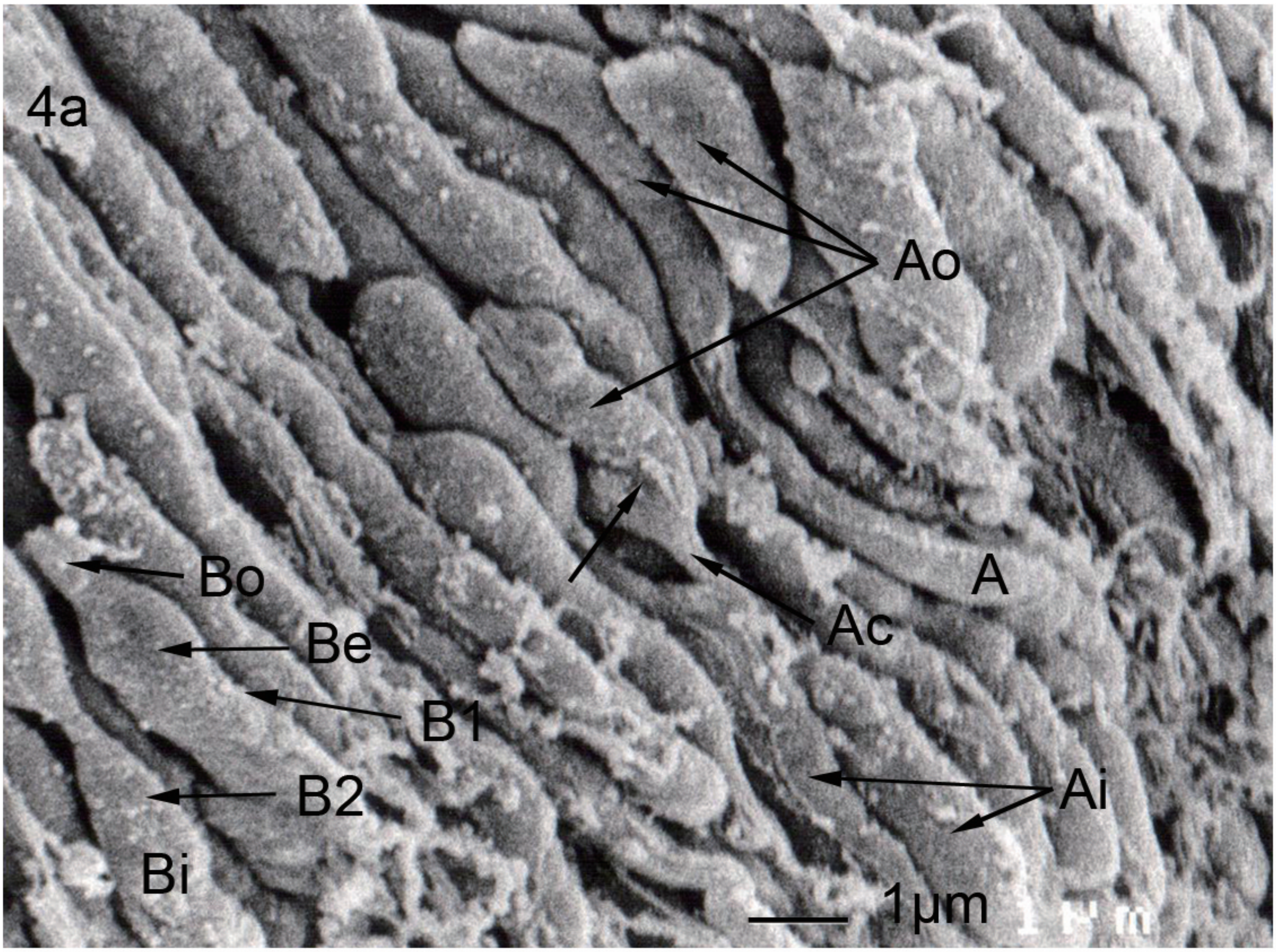
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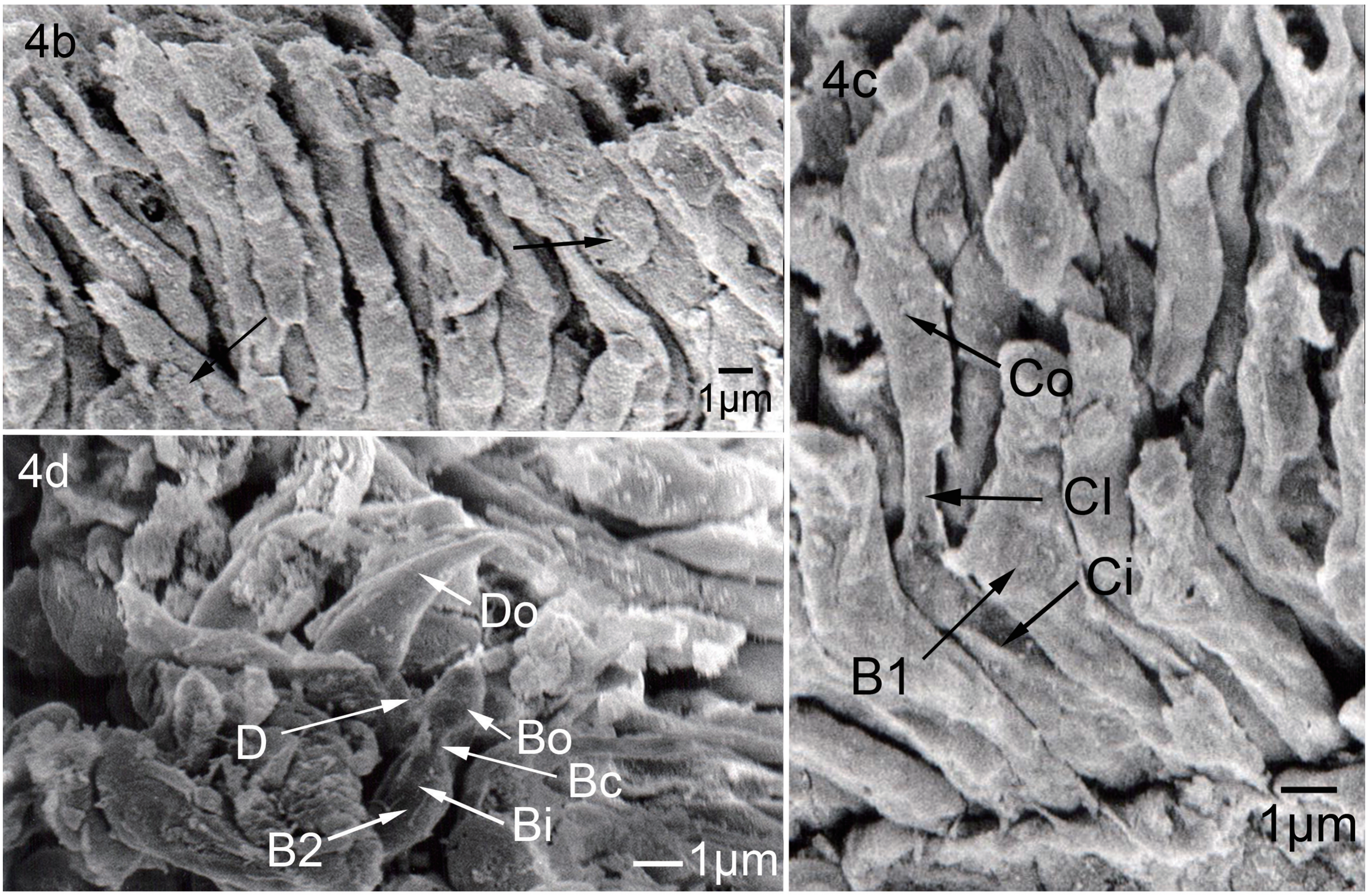
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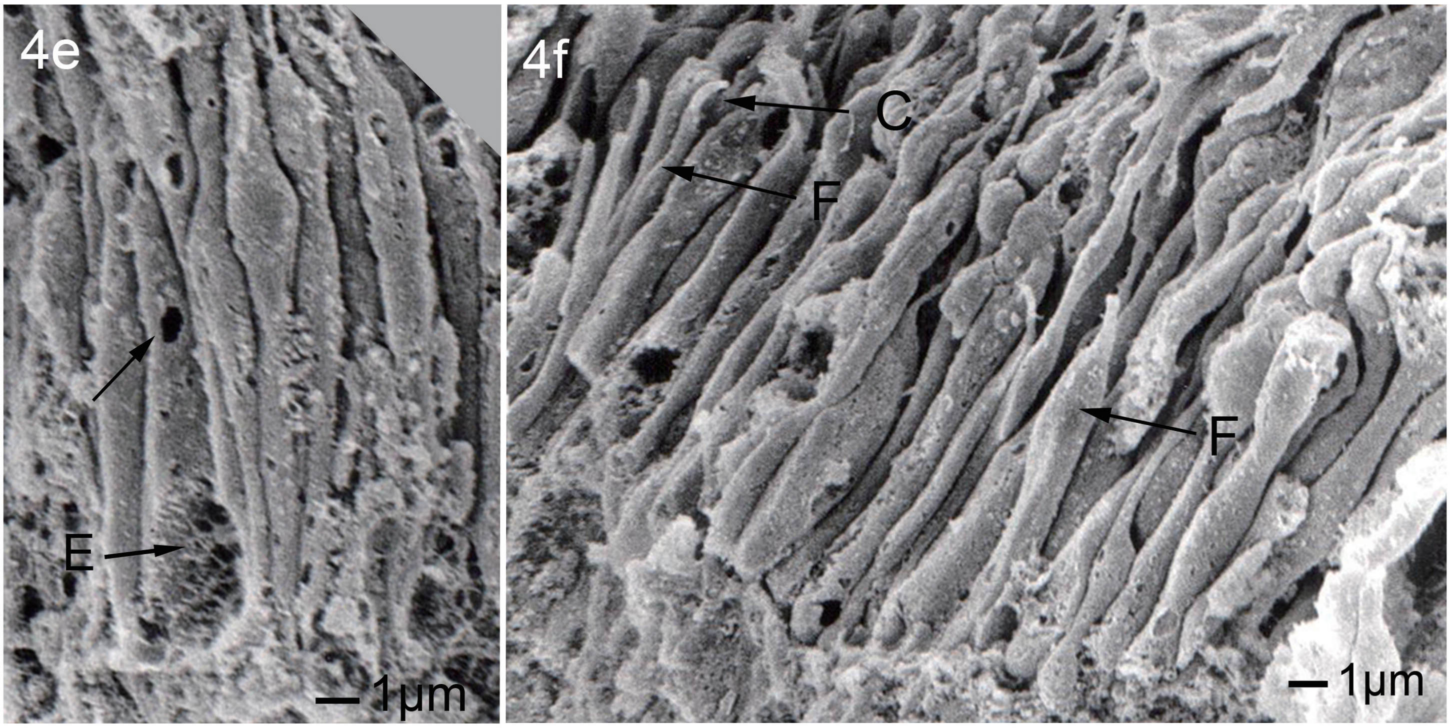
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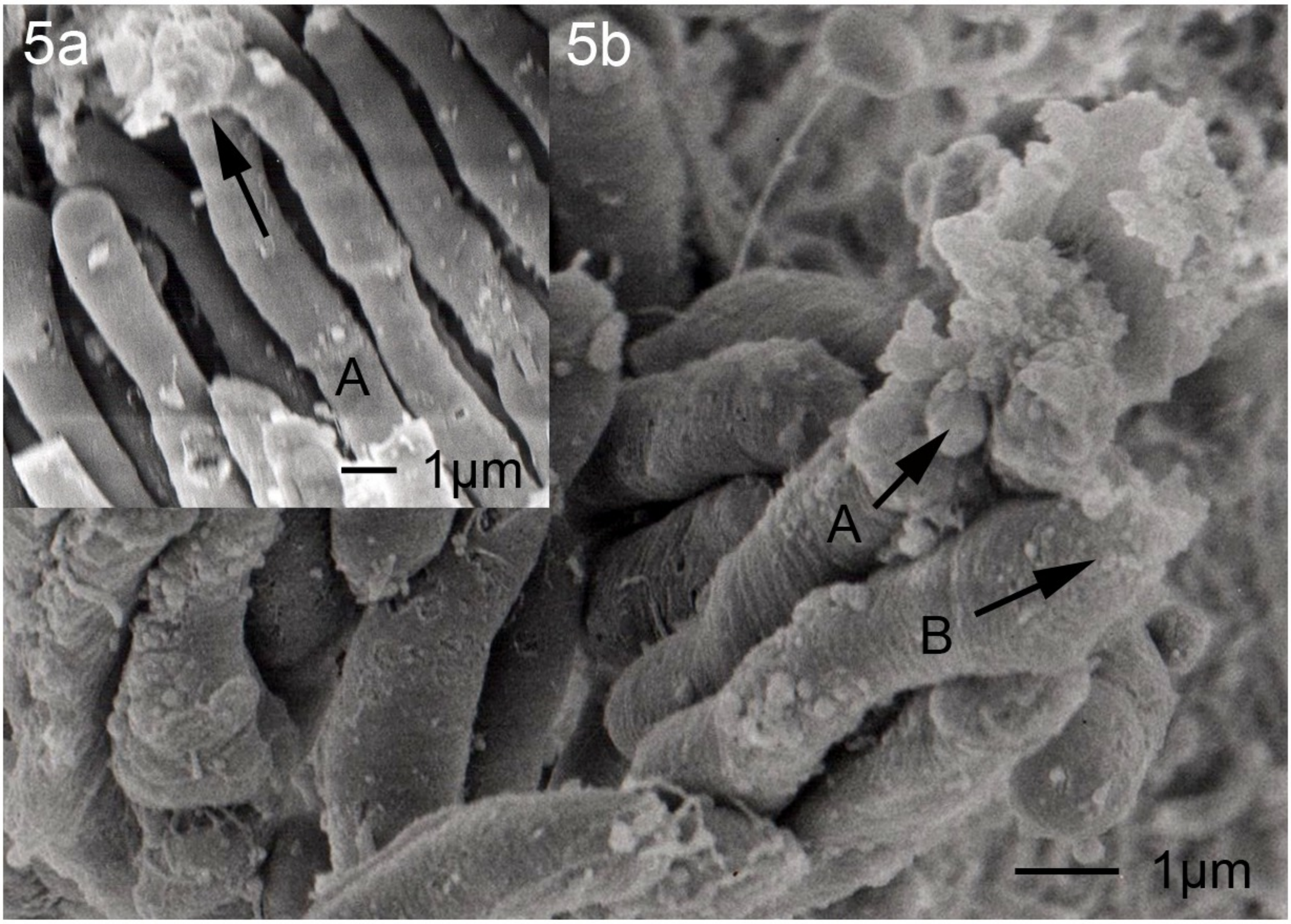
5a
A
1µm
5b
A
B
1µm

Total percentage of rod cells in respective areas of the retina