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Three-Dimensional Reconstitution of Cone Arrangement on the Spherical Surface of the Retina in the Medaka Eyes

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ABSTRACT—In the retina of the medaka four kinds of retinal cone photoreceptor cells are arranged in a simple, repeating organizational pattern known as a square mosaic. We found that the distribution of cones in the retina could be easily detected by autofluorescence-emission from the photoreceptor cells without any staining. In tangential sections of the retina, cones were located at a specific position in a crystalline lattice as follows: Double cone pairs display a zigzagging appearance, oriented roughly 90-120 degrees to one another, and single cones were in the center of the square consisting of four double cone pairs. In order to determine the continuity of this regular arrangement on the spherical surface, the distribution of this cone mosaic pattern was examined in central, dorsal, ventral, nasal and caudal areas of the retina. The regular arrangement of cones was confirmed in the whole retina. Double cones and single cones are in their respective lines and these lines form a lattice-work. As a result of reconstructing these arrangements on the retinal hemisphere, the lines of this lattice-work of cones were found to be orthogonal to the retinal margin, radiating from the center of the retina-like meridians, and parallel to the retinal margin forming concentric circles that is reminiscent of a longitudinal and a latitudinal lines of a terrestrial globe. This construction of the cone arrangement in whole retina of the medaka was consistent with maintaining a rectangular mosaic in growing retina with newly produced cells only in a marginal cell proliferating zone.

INTRODUCTION

The neural retina of teleosts is a highly organized structure, made up of a layer of photoreceptors and a layer of nervous tissue. In the layer of photoreceptors there are two types of photoreceptor cells, namely rods and cones. In most teleosts single and double cones, situated in a single layer, are arranged in a simple, repeating organizational patterns, known as a cone mosaic (Lyall, 1957; Engstrom and Ahlbert, 1960, 1963; Ahlbert, 1976). The distribution of cones is not always uniform throughout the retina. Regional differences of the cone arrangement are usually found in the distance of neighboring cones or in the cell densities. Moreover, some species of fish, for example pike, show intraretinal differences in cone mosaic pattern (Lyall, 1957; Engstrom and Ahlbert, 1960, 1963; Kawamata, 1994).

In the retina of the medaka, there are four morphological types of cones; a short single cone (SS), a long single cone(LS), a short member (SD) and a long member (LD) of a double cone pair (DC pair). These cones are packed closely and the repeating organizational pattern of cones are called the square mosaic pattern. In the retina of the medaka, cones are lined in two types of rows, rows of zigzagging double cones and rows of alternating short and long single cones. All rows are arranged to form a lattice-work and rows of single cones are separated by a row of double cones. According to a report of the electron microscopic observations (Ohki and Aoki, 1985), eight cones of four DC pairs surrounding SS form a cross and those surrounding LS form a rhombus (Fig. 1 bottom). The regular pattern of cones was supposed to be the same in most regions of the retina (Ohki and Aoki, 1985). The distribution of cones in the medaka is a two-dimensional lattice on hemispherical surface. So the spatial position of cones can be projected on the retinal hemisphere. Since the distribution of the cone arrangement is very simple in the retina of the medaka, it is highly suitable to analyze the mechanism of the cell organization in the tissue.

Assuming that each cone has a specific opsin for color vision, the regular arrangement of cones may reflect the functional arrangement of cones. Teleost fish eyes grow throughout life without compromising visual performance of the animal (Hairstone *et al.*, 1982; Ohki and Aoki, 1985, 1991). Retinal surface is expanded by stretching the existing retina and

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by generation of new tissue. In the postembryonic teleost retina, new cells are produced in the annular germinal zone (the marginal germinal zone) located at the junction between neural retina and iris epithelium (Lyall, 1957; Johns, 1977; Johns and Easter, 1977). As cells proliferate, their postmitotic daughter cells remain central to the retinal margin, and the proliferative stem cells are displaced centrifugally as a part of the ever-increasing retinal perimeter. In the retina of the medaka the annular germinal zone in the adult was also detected with anti-PCNA (proliferating cell nuclear antigen) antibody (Negishi *et al.*, 1990). So, the medaka with expanding retina must maintain the regular arrangement of cones by cell-proliferation only in the restricted germinal area.

The cone mosaic pattern in the retina of the medaka was regarded as the most suitable structure to clarify the mechanism of cell-organization. The present work was performed to analyze a spatial construction of cone mosaic pattern in the medaka. We examined the lattice-like arrangement of cones in five distinct region of the eye and projected these arrangements on the retinal hemisphere. The minimum unit of the lattice, consisting of four neighboring DC pairs, is square in the central region while that in the peripheral region, the dorsal, ventral, nasal and caudal regions, is a rectangle. Pasting these arrangements on retinal hemisphere in their respective regions, one side of the rectangle in peripheral regions is always found to be orthogonal to the retinal margin and another side is parallel to the retinal margin, and the side orthogonal to the retinal margin is always shorter than the side parallel to the retinal margin. These suggest that the rectangles are chained together to form a concentric circles. So the repeating pattern of the cone arrangement is supposed to be continued in two directions, radiating from the center of the retina and parallel to the retinal margin to form concentric circles.

MATERIALS AND METHODS

Animals

An orange-red variety of the adult medaka, *Oryzias latipes* (1.5-3.5 cm in body length) was purchased from a local breeder. Larvae(fry) and young fish are raised in our laboratory. The fish were breded in the physiological solution (PS; Yamamoto, 1949). A 24-hr light-dark cycle (LD 14:10) was maintained. It has been well known that retinomovements occur in synchrony with changes in ambient light levels. In the light condition, the outer segments of rods lie more sclerally in the retina while cones were present forward in the inner layer of the retina. On the other hand, rods were almost in the same layer as cones in the dark-adapted eye. So fully light adapted fishes were used in most cases of the present experiments.

Histochemistry

Observations of cone mosaics with autofluorescence

Whole larvae and young fish were fixed in Bouin's fixative for one day at room temperature (24°C). Removed adult heads were fixed for more than one day and washed in 70% ethanol several times; dehydrated through graded butanols; and embedded in paraplast or Technovit resin (Technovit 8100; Wehrheim, Federal Republic of Germany). These samples were sectioned at a thickness of 5 μm and sagittal, transverse and coronal sections of the whole head were prepared. Autofluorescence of tissues was examined with a fluorescence microscope (BH2-RFK, Olympus, Tokyo) equipped with blue excitation filters.

Observations of cone mosaics with a monoclonal antibody

The heads of adult fish were fixed overnight with 2% paraformaldehyde (PFA) in PBS and embedded in the Technovit resin as described above at 4°C. After incubation with 1% BSA-PBS with 0.5% Triton X-100 for 30 min, sections of 5 μm were incubated in a primary monoclonal-antibody (Rh29 which labels bovine rhodopsin; Tokunaga *et al.*, 1989) for 2 hr and a secondary antibody conjugated with fluorescein isothiocyanate (FITC anti-mouse IgG, Tago Inc; 1:200 dilution) for 30 min at 25°C. Counter-staining by 0.01% Evans Blue in PBS for 10 min, slides were cover slipped with PermaFlour and observed by fluorescence microscopy.

Observations of cellular proliferation

In the BrdU-label experiments, fish were injected with approximately 20 μl of 10 mM 5-bromo-2'-deoxyuridine (BrdU; Sigma) and 1 mM 5-fluoro-2'-deoxyuridine (Larison and BreMiller, 1990) intravitreally. After injections, the fish were kept in the dark condition for 4 hr and then, the heads were dissected, fixed, embedded and sectioned as described above.

Then coronal sections of 5 μm were treated with 4 N HCl for 30 min. The sections were neutralized with NaOH in PBS. After incubation with 1% BSA-PBS with 0.5% Triton X-100 for 30 min they were incubated with a primary anti-BrdU antibody (Anti-BrdU; BECTON DICKINSON; 1:50 dilution) for 2 hr then treated in the same steps as described above.

Morphological analysis

Estimating a width and a length of the square consisting of four DC pairs, an average of the distances between neighboring DC pairs was worked out by measuring distance between randomly sampled neighboring DC pairs on microphotographs that were placed on the opened retina as shown in Fig. 3. Cones in a randomly sampled rectangle were counted on the microphotograph, and an area of the rectangle was calculated by multiplying the length by the width of the rectangular area which were measured on the microphotograph. The cell density was calculated by dividing the number of cones by the rectangular area. To estimate the total cone number of developing retinas, the area of the retinal hemisphere was calculated with a diameter of the eye regarding the structure of the retina as the hemisphere. Diameters of the developing eyes were measured on the sections under microscopic observation. The number of cones was estimated by multiplying the area of retinal hemisphere by the cell density of the central region in the retina.

RESULTS

Autofluorescene analysis of the retina

The cell distribution in the retina of the medaka could be easily detected by autofluorescene of cells. The retina of adult medaka contains five types of photoreceptors; the rods plus four cone classes. Cones were distributed regularly in pattern known as square mosaic. Since cones are interdigitated with the pigment epithelium, this mosaic pattern has been hardly analyzed with a conventional light microscope at a low magnification. But under fluorescence microscopic observation, the localization of cells containing cones in the retina could be detected and we could discriminate photoreceptors based on morphological differences (Fig. 1 radial). The inner segments of cones were arranged closely and their outer segments were interdigitated with the pigment epithelium. Rod outer segments were placed more sclerally and separated from the cone pho-

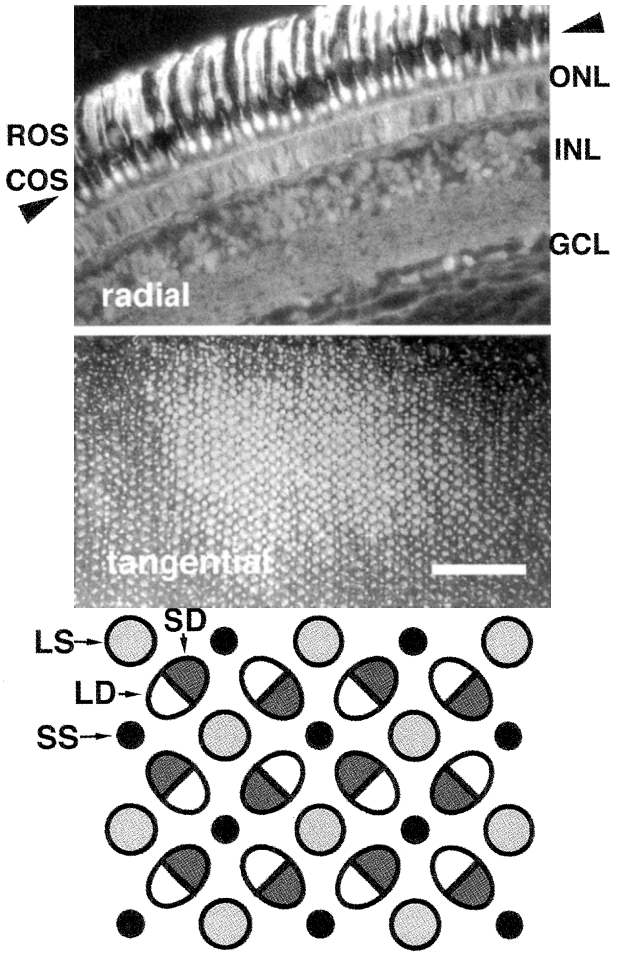

Fig. 1. The figures show photomicrographs of radial (upper) and tangential (middle) sections from an adult medaka eye by the fluorescence microscopy. In the radial section, the retinal pigment epithelium exists upperside and the lens is located downward. The tangential section was sectioned at the level indicated by arrow heads. The bottom schematic representation shows the cone mosaic pattern of the medaka. SS, short single cone; LS, long single cone; SD, short member of double cones; LD, long member of double cones; ROS, rod outer segment; COS, cone outer segment; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar, 50 μm.

toreceptor layer. We could discriminate each cell types of cones based on their positions in the mosaic pattern and confirm the mosaic pattern under the observations of broad field by autofluorescence of the cells against the pigment layer as a background (Fig. 1 tangential). The rows of single cones, alternating short and long single cones by turns, were detected as narrow streams while the pairs of double cones zigzagging in the rows were detected as broad streams. These two types of rows are parallel and alternating. And single cones are in the central region of the square, consisting of four pairs of double cones. This method of observing samples by autofluorescence was convenient and useful to analyze the cone distribution on the spherical surface of the whole retina.

Opsin distribution in the cone mosaic

The relationship between the morphological pattern and the functional arrangement of cones were examined by immunohistological method with Rh29 monoclonal antibody raised against bovine-rhodopsin (Tokunaga *et al.*, 1989). In the retina of the medaka, Rh29 antibody strongly bound to the outer segment in the short member of the double cones (SD) only (Fig. 2a). It was also clear in the tangential section that Rh29-positive cells are SD. SD cells were Rh29-positive and lined up zigzag in the cone mosaic pattern (Fig. 2b). On the other hand, Rh29 antibody shows very low affinity to the rod outer segments, almost similar to non- specific binding. These results indicate that SD has a specific type of opsin different from opsins in other types of cones and from rhodopsin in rod. Therefore, the morphological pattern of cones may directly reflect the functional arrangement of cones.

Three dimensional distribution of cone mosaic

The distribution of the cone arrangement was observed by their autofluorescence in the tangential section of the retina. Each photograph in Fig. 3 shows the distribution of cones in five distinct regions, central, dorsal, ventral, nasal and caudal region of the retina. These photographs were rearranged correctly on a schematically opened retina. The central area of the retina was projected in the sagittal sections, the dorsal and ventral areas were projected in the coronal sections and the nasal and caudal areas were projected in the transverse sections of the heads. Four neighboring double cone pairs formed squares in the central retina, and they formed rectangles in the peripheral regions. In order to make the presentation of rectangles easier, rectangle is defined as the inset in Fig. 3. The short sides of the rectangles in the peripheral regions were the sides formed by cones radiating from the central retina, and the long sides of the rectangles were parallel to the marginal line of retinal hemisphere (Fig. 4). Inferring from these characters of rectangles, these rectangles may be beaded to form a choker. Thus we deduced that the cones were arranged on the spherical surface such that rows of cones radiated from the center of the retina, and other type of cone rows are perpendicular to the radial direction to form concentric circles (Fig. 3b). In some species with uniform arrangement of cones, for example the green sunfish, cone densities were changed across the retina (Cameron and Easter Jr., 1993). In the retina of the medaka there are also regional differences in cone density in that the DC pair density is lower in the dorsal area than in other areas (Fig. 4). These differences of DC pair densities were reflected by the differences in size and shape of the rectangle square in each region.

Proliferation of the cone cells during expansion of the retina

The square mosaic pattern of the adult medaka was always observed in growing retina since the medaka hatched. The number of cones increased as the medaka grew (Table 1). Then we verified the distribution of proliferating cells in the retina by BrdU-labeling (Fig. 5). BrdU-positive cells in the retina

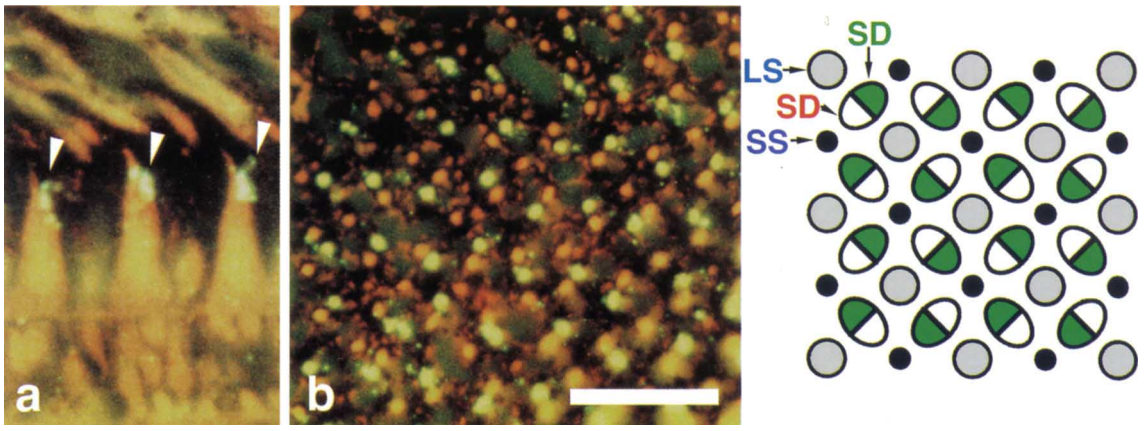

Fig. 2. Rh29-antibody staining of the retina. Rh29-positive cells are yellowish-green while most cells detected by autofluorescence are red or orange. (**a**) In the radial section the outer segment in the short member of double cones was only immunoreacted with Rh29 antibody (arrow head). (**b**) Patterning in cone mosaic as revealed by Rh29-antibody staining. Rh29-positive and negative double cone cells are zigzagging alternatively in the rows of DC. Scale bar: 25 μm.

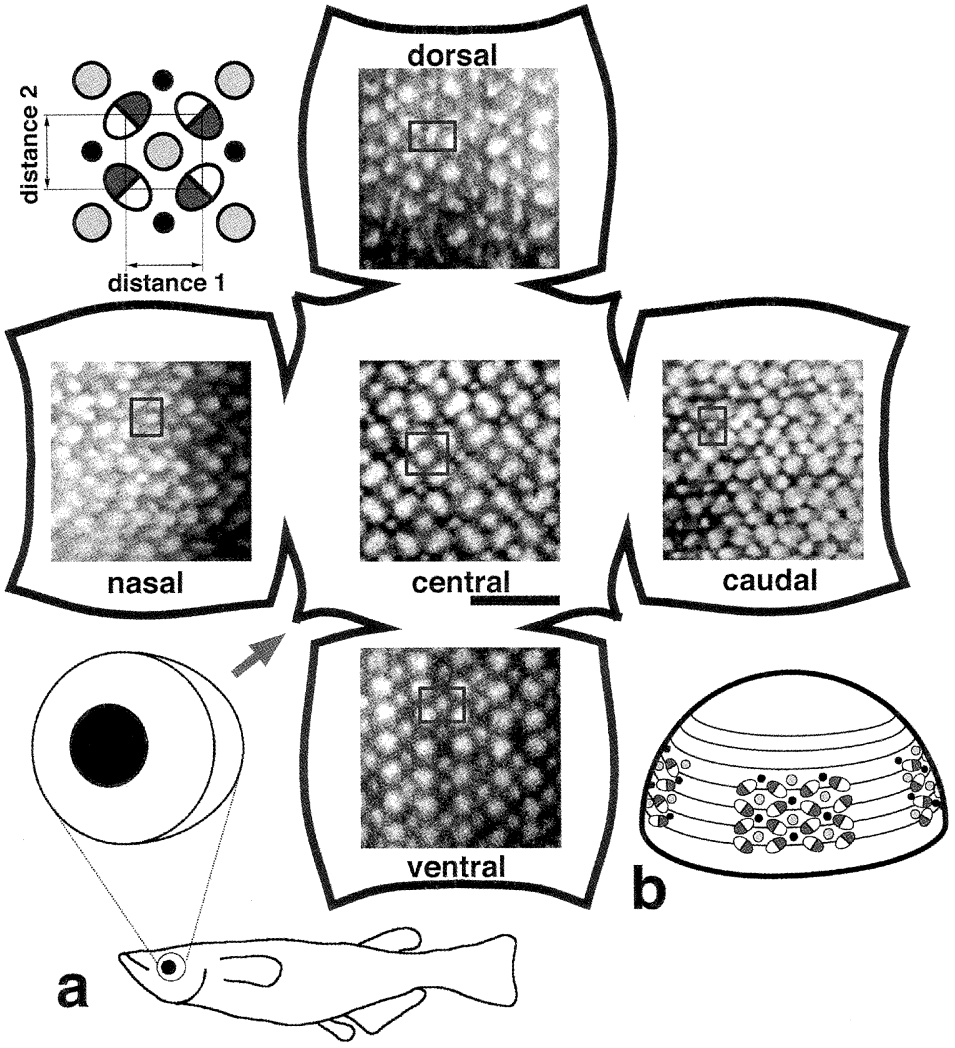

Fig. 3. (**a**) Photographs of the arrangement of cones in central, dorsal, ventral, nasal and caudal region of the retina in an opened retina of the left eye. The rectangles consisting of four neighboring DC pairs are given by gray line on photographs. Scale bar: 25 μm. (**b**) Schematic representation of the cone arrangement on the spherical surface.

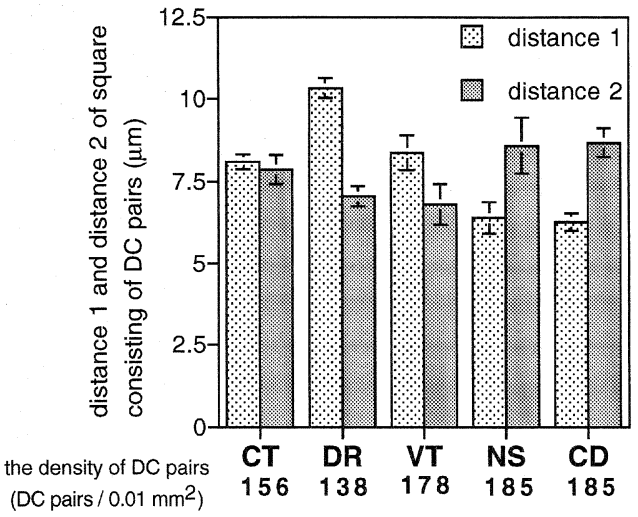

Fig. 4. Histogram showing spacing of DC pairs in five regions of the retina. CT, central; DR, dorsal; VT, ventral; NS, nasal; CD, caudal region of the retina. Defining as the inset in Fig. 3, distance 1 means the distance between neighboring DC pairs in the horizontal lines of cones and distance 2 means the distance between neighboring DC pairs in the perpendicular lines of cones on photographs in Fig. 3. The numbers below each column are the planimetric density of DC pairs (DC pairs/0.01 mm^2).

Table 1. Increase in cone number of growing retina

stage	diameter of the eye (μm)	total cone number ($\times 10^3$)
hatching *	402.6 ± 11.8	1
young fish **	852.5 ± 7.0	10
adult fish ***	3180.4 ± 52.5	300

* 0 day after hatching.
** Approximately 1 month old fish.
*** More than 1 year old fish.

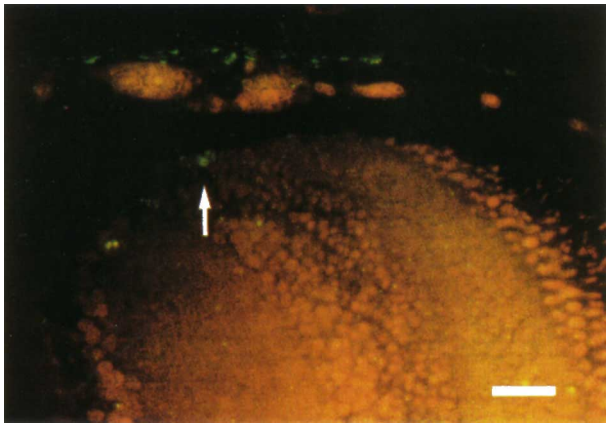

Fig. 5. Radial section of the retina labeled with anti-BrdU antibody. Fish were injected with 5-bromo-2'-deoxyuridine (BrdU) 4 hr before sacrifice. Actively dividing cells are yellowish-green (arrow).

were observed only in marginal region of the retina. These results implied that the new cones were derived mainly from the marginal retina in growing eyes of the medaka, and adding new cones to the marginal retina formed a new concentric circle of the lining cones. Thus, it was consistent with our speculation that cones were lined up in concentric circles on the spherical surface. The continuity of the lines of DC pairs on the retinal hemisphere was expressed as a frame model in Fig. 6. Bold lines show the lines of cones parallel to the retinal margin and narrow lines show the lines of cones radiated from the center. Intersecting points show each position of double cones. If the cones were arranged in concentric circles, the number of cells on a newly added circle must be increased to keep regular distance between neighboring cones. Our reconstitution indicates the existence of regulatory site of a cell number in which new spokes of cells radiating from the center of the retina were intercalated (surrounding by the gray line in Fig. 6), and the irregularity in the cone mosaic may be a regulatory site (Fig. 6, asterisks).

DISCUSSION

In the retina of the medaka, there are only four kinds of cones and it was assumed that cones are distributed to form a repeating organizational pattern throughout the retina (Ohki and Aoki, 1985). In the present study, we attempted to clarify the continuity of the cone mosaic pattern.

In the teleost retina, cones functionally arranged like tricolor grids in a TV screen. In zebrafish and goldfish, for example, the different spectral / morphological subtypes of cones are located at specific positions in the arrangement (Nawrocki *et al.*, 1985; Raymond *et al.*,1993). Medaka was shown to have four morphological types of cones (Ohki and Aoki, 1985) and was thus expected to have cDNA corresponding to red, green, blue and UV opsins (Hisatomi *et al.*, 1994). The outer segment of SD in the medaka was immunolabeled with Rh29 antibody, an anti-bovine-rhodopsin antibody. The amino acid sequence of Rh29 binding site to bovine-rhodopsin was most homologous to the deduced amino acid sequence of the green visual pigment in the medaka (unpublished observation). Moreover, λ_{max} of the green opsin is more similar to λ_{max} of the rhodopsin than other opsins in many species (Kaneko, 1989) and several rhodopsin antibodies crossreact with green cones (Raymond *et al.*, 1993). Therefore, SD in the medaka may be a green-light photoreceptor cell in the medaka. The correspondence of the SD arrangement to the Rh29-positive cell arrangement implies that four morphological types of cones may correspond to four spectral subtypes respectively.

We have developed a very convenient method for analyzing the cone cell distribution in the retina of the medaka. We can detect cone cells without staining by their autofluorescence under a fluorescence microscope equipped with a blue excitation filter. It was confirmed that the pattern and composition of cone mosaic was uniform throughout the retina of the medaka both in the peripheral and in the central regions. With this method that made it possible to observe the

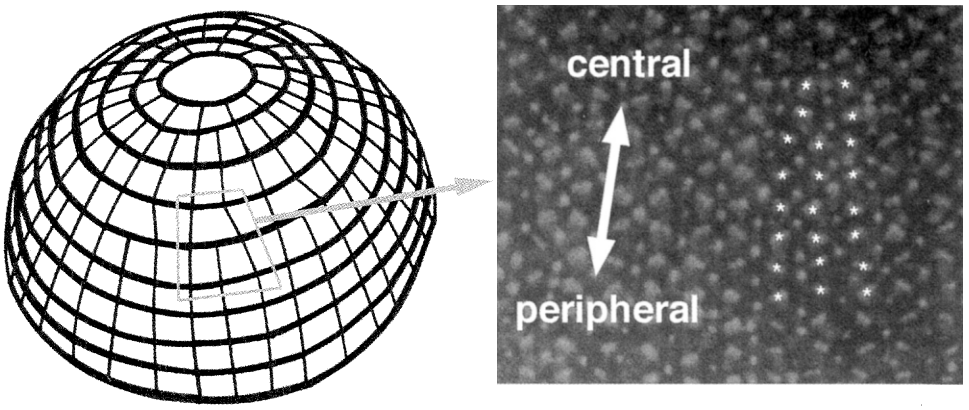

Fig. 6. The left schematic presentation shows the theoretical reconstitution of cone rows on whole retinal hemisphere. Our model indicates the existence of regulatory sites that control cell number (given by gray line on the scheme). And irregularity in the cone mosaic (asterisks) may be consistent with the location of the regulatory site.

cone distribution in a broader field, we could confirm the cone distributions as alternating stripes and could project the plane distribution of cones onto the retinal hemisphere.

There were regional differences in the style of rectangles formed by four neighboring cones in the medaka. The rectangles of central region of the retina were square but they were not square in the dorsal, nasal, ventral and caudal areas. In these four distinct regions, the distances between neighboring double cone pairs aligned perpendicular to the marginal line of retinal hemisphere were nearly equal. In the peripheral areas, a zigzag of double cones parallel to the retinal margin formed an angle of 100-110 degrees. These geometrical similarities suggest that the rectangular unit of four double cone pairs repeats to form concentric circles. So it was expected that the continuity of rows of cones in the whole retina were arranged in lines radiating from the central region and in the lines of concentric circles.

Our observations also show regional variations in the cone cell density. The differences in the cell density were reflected mainly by the cell distance of neighboring cones aligned parallel to the retinal margin. The cell density was more condensed in the ventral hemiretina than the dorsal hemiretina (Fig. 4). The medaka usually live in shallow water and they must look more carefully up than down, so visual acuity in the ventral hemiretina is expected to develop more than that in dorsal hemiretina. This was consistent with the topological variations in planimetric density.

The repeating cone mosaic pattern and the expected distribution of cones are reminiscent of the stitches of a knit cap. When knitting a cap, a repeating distance of mosaic unit reflects planimetric densities. When we knit loosely, the stitch density decreases while the stitch density increases when we knit tightly. To form a hemispherical shape, the stitch number on each concentric circle must be regulated as the length of its circumference becomes longer. In the scheme of Fig. 6, stitches are shown as intersecting points and lines of stitches are shown as frame lines. In the regulated region of the stitch number, new spokes of stitches which are radiated from the center of the hemisphere are intercalated (the scheme in Fig. 6), and the intercalations of a new line of cones in the cone arrangement shown in Fig. 6 may be a regulatory site of the cell number on concentric circles.

The cone mosaic is continued in an orderly manner into the inner layers of the retina (Engstrom, 1963; Wagner, 1975). The regular conformations of cone mosaic patterns may be efficient hardware for information management. Therefore irregularity of the cone arrangement must be dispersed over the whole retina because localization of irregularity would disrupt the cone mosaic and cause problems in processing visual information. Therefore regulatory sites of the cone number in the scheme of Fig. 6 were dispersed on concentric circles, and intercalating new spokes of cones could be observed throughout the retina like the scheme in Fig. 6. This supports our speculation of the cone distribution.

Growing retinas of teleosts expand mainly by adding new lines of cells to the marginal line in the peripheral region. In the retina of goldfish, for example, new cells are added in concentric rings (Johns, 1977). In the retina of the medaka, proliferating cells were detected only in the marginal germinal zone as shown in Fig. 5. On the other hand, it has been known that constant visual acuity in the medaka is maintained throughout life and the distance between neighboring double cones widens but not as much as their eye's grow (Ohki and Aoki, 1985, 1991). To maintain constant distance between cones, the new rows of cones must be intercalated into the present cone mosaic pattern and the number of the cells in inner circles must increase as the retina grows. A source of new cells is also necessary. We could not find proliferating cells in the inner circles of cones with BrdU. Two possibilities could explain this. One reason might be that very few proliferating cells are necessary to maintain the distance between cones, perhaps so few that we could not find these cells. Another reason might be that proliferating cells in inner circles were dividing so slowly that we could not detect the S-phase of these cells with BrdU injections.

In the growing retina of the medaka, new cones were

added to the marginal retina. Since the cells in the last line of generated retina may control the fate and position of new additional cells in the next line, the mechanisms of organizing the repeating pattern in the cone arrangement could be accounted for by hypothetical molecular mechanisms, for example, a reaction-diffusion system or a lateral inhibition system (Meinhardt, 1989; Honda, 1994).

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