CHAPTER 5

THE ARRANGEMENT OF L AND M CONES IN HUMAN AND A PRIMATE RETINA

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Introduction

The relative numerosities and the spatial arrangement of the long-wave (L) and middle-wave (M) cones in the human and primate retina have long been debated. This topographical organization of cones is fundamental to our understanding of visual sensitivity and of colour vision. To date, owing to the very similar structure of the L and M opsins (Nathans *et al.* 1986), it has not been possible to label the two spectral classes of cone independently, and thus indirect methods have had to be employed. In humans, psychophysical estimates (Vos and Walraven 1971; Cicerone and Nerger 1989; Pokorny *et al.* 1991) suggested a preponderance of L cones, with an average L : M cone ratio close to 2 : 1, but with considerable variation between individuals, the estimated ratios ranging from about 0.3 : 1 to 3 : 1 (Rushton and Baker 1964). More recent findings, including ERG measurements (Carroll *et al.* 2000; Dobkins *et al.* 2000), suggest a similar average ratio and confirm the individual variability, with ratios as high as 12 : 1. Adaptive optics have made possible the direct imaging of the photoreceptor mosaic for spatially localized retinal densitometry and have allowed the L : M ratio to be estimated by differential bleaching (Roorda and Williams 1999; Brainard *et al.* 2000; Roorda *et al.* 2001). For two individuals this method gave ratios of 1.2 : 1 and 3.8 : 1. The distribution of L and M cones appeared random.

Estimates of the L : M cone ratio have also been made from the levels of mRNA, either in whole retina or in regionally located pieces of retina. In human retinae the ratio appears to increase from central retina to periphery, with a ratio in the fovea of about 1.3 : 1 rising to between 3.5 : 1 and 5 : 1 in the mid periphery (Yamaguchi *et al.* 1997; Hagstrom *et al.* 1998, 2000). There was considerable variation between individuals. The suggestion that the relative number of L cones increases towards the periphery (Hagstrom *et al.* 1998) implies that the cone-rich rim of the retina at the *ora serrata* may be dominated by L cones.

In the human retina a stochastic process is thought to determine which form of long- or middle-wave opsin will be expressed in a given cone. The L and M opsin genes are

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located on the X-chromosome in a tandem array, with one L opsin gene followed by two (or more) M opsin genes (Nathans *et al.* 1986). A locus control region (LCR), upstream of the first opsin gene, is thought to bend back to interact with a promoter region at the beginning of one of the opsin genes (Wang *et al.* 1992, 1999). Only the gene that is coupled to the LCR is favoured for expression. The probability of a given gene being expressed may depend on (i) the exact sequence of the promoter region and (ii) the distance of the gene from the LCR (Winderickx *et al.* 1992). In human females, the system of random X-chromosome inactivation is superposed on the stochastic choice of opsin gene within the expressed chromosome; and thus there is the possibility of more than two types of long-/middle-wave cone within a given retina (Mollon and Jordan 1988).

The organization of the L and M gene array in nonhuman Old World primates is similar to that in humans (Dulai *et al.* 1994, 1999), though normally there is only a single copy of the M gene (Onishi *et al.* 1999). Thus it might be expected that the arrangement of cones within Old World primates would be identical to that in humans. However, spectral sensitivities determined by flicker ERG for chimpanzees (Jacobs *et al.* 1996) suggested a ratio of about 1.3 : 1, somewhat lower than in humans. A lower ratio has also been reported for catarrhine monkeys from direct microspectrophotometric measurements (Bowmaker *et al.* 1991; Mollon and Bowmaker 1992), from flicker ERG (Jacobs and Deegan 1997), from red–green equiluminance matches for magnocellular units (Dobkins *et al.* 2000) and from L and M cone inputs to H1 horizontal cells (Dacey *et al.* 2000). Spatially localized retinal densitometry gave a ratio of 1.4 : 1 in an individual macaque (Roorda *et al.* 2001). Estimates of the L : M cone ratio made from mRNA levels (Deeb *et al.* 2000) yielded a value for macaques of about 1.6 : 1, lower than the value for humans of 4 : 1.

By contrast, in the case of most platyrrhine primates, such as marmosets, there is only a single opsin gene on the X-chromosome, but this locus is polymorphic in many species of New World monkey. In females heterozygous at the single locus, the random process of X-chromosome inactivation will ensure that only one or other opsin allele is expressed in a given cone (Mollon *et al.* 1984). Thus, in a trichromatic female, if X-chromosome inactivation is strictly random, the ratio of the two classes of cone in the red/green spectral region should be close to unity.

We have addressed the question of the numerosity of L and M cones in the human fovea directly by microspectrophotometry of individual cones, in a similar manner to that used previously for talapoin monkeys (Mollon and Bowmaker 1992). However, in the case of *post mortem* human tissue, where the photoreceptors are fully bleached, we have reconstituted the cone visual pigments with a synthetic retinaldehyde. We have also attempted to examine cones at the *ora serrata* and have analysed the foveal array of a trichromatic female New World monkey, *Callithrix jacchus jacchus.*

Methods

Tissue

Human retinal tissue was obtained from the eye bank of Moorfields Eye Hospital. For the successful preparation of foveal cone arrays, we have found that the tissue has to be less than 48–72 hours *post mortem* and from donors less than about 55–60 years old. From the eye cup, a small disc about 2 mm diameter centred in the macula was removed, along with a section of the *ora serrata* from the nasal region. In these preparations, the photoreceptors were fully bleached, since it was not possible at any stage *post mortem* for the eyes to be maintained in the dark. A dark-adapted retina from a female marmoset was obtained about 5 hours *post mortem*, by courtesy of Dr Martin Tovée of Newcastle University.

Reconstitution of visual pigments

We have developed a protocol for reconstituting visual pigments with synthetic retinaldehyde isomers, using the goldfish retina as a model system (Parry and Bowmaker 2000). Ideally, reconstitution would be carried out with 11-*cis* retinal, but because 11-*cis* retinal is not available commercially and because of the relatively large quantities necessary to reconstitute whole pieces of retina, we have used 9-*cis* retinal. The goldfish was chosen as a model system because it has, in addition to rods (λ_{max} 522), four spectral classes of cone extending throughout the spectrum with λ_{max} at about 380, 450, 535, and 620 nm. These pigments are all porphyropsins, based on vitamin A_2, and were reconstituted with both 11-*cis* retinal$_1$, to form rhodopsins, and 9-*cis* retinal$_1$, to form isorhodopsins (Knowles and Dartnall 1977). 11-*cis* retinal was a gift from Dr Rosalie Crouch and 9-*cis* was obtained commercially (Sigma).

Retinal tissue was incubated at 4 °C for about 3 hours, in a solution containing lipid vesicles incorporating retinal. Retinal and phosphatidylcholine were each dissolved in hexane and aliquotted together, then evaporated to dryness under argon and stored at −20 °C. Vesicles were formed by the addition of saline to an aliquot, vortexing for 10 min, then sonicating on ice for 3 min (Parry and Bowmaker 2000). The vesicles were present in excess to ensure complete reconstitution of visual pigments, but typically contained 60 μg 9-*cis* retinal and 1.5 mg phosphatidylcholine in 150 μl of saline.

Microspectrophotometry

After incubation, small pieces of retinal tissue were mounted on a cover slip and squashed under a second cover slip. Microspectrophotometric recordings were made in the conventional manner using a Liebman dual-beam microspectrophotometer (Liebman and Entine 1964; Mollon *et al.* 1984; Bowmaker *et al.* 1991). Spectra were recorded at 2-nm intervals from 750 to 350 nm and from 351 to 749 nm on the return scan. The outward and return scans were averaged. A baseline spectrum was measured for each cell, with both beams in an unoccupied area close to the cell, and this was subtracted from the

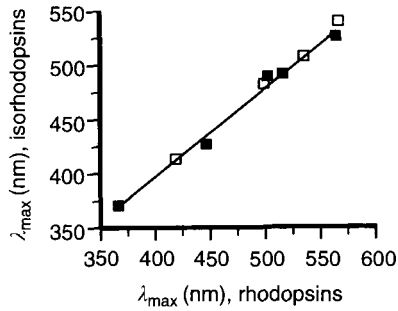

Figure 5.1 Correlation of λ_{max} of visual pigments based on 11-*cis* retinal (rhodopsins) and 9-*cis* retinal (isorhodopsins). Filled squares, the rod and four cone pigments from the goldfish (from Parry and Bowmaker 2000); open squares, the rod and three cone pigments from humans.

intracellular scan to derive the final spectrum. Two baseline scans were recorded for each cell and averaged. Cells were not routinely bleached, so as to avoid the possibility of low levels of bleaching in neighbouring cells in the array.

From the λ_{max} of the reconstituted goldfish pigments, it was possible to establish a linear relationship between the λ_{max} values of the rhodopsins and isorhodopsins (Parry and Bowmaker 2000). In the case of the human tissue, the L and M cone and rod pigments behave in an identical manner, exhibiting the same linear relationship (Fig. 5.1). The λ_{max} values of the L and M cones are displaced from about 565 and 535 nm to about 540 and 510 nm in the isorhodopsin form. Since we are interested only in whether an individual cone is L or M (or S), the precise λ_{max} is not important, but in all cases, a given cone could be assigned to either the L or the M class.

Results

Human tissue—foveal region

From two individuals, small patches of cone arrays from regions close to the fovea were successfully measured. In two further individuals, although clear arrays were not observed, sufficient cones in close proximity were recorded to estimate the ratio of L : M cones.

In a foveal array from a male (H24, aged 56) 44 cones were identified, one of which was an S cone (Fig. 5.2). Five additional cones, in close proximity to the array were also measured, together with eight rods. The cones were clearly divided into two classes with a ratio of L : M cones of 1.2 : 1. It was noticeable that the S cone did not disrupt the array and that two rods measured in the area of the array were in a slightly different focal plane of the preparation and did not fall within the array.

From a second individual (H25, a female aged 48) a less precise array was observed from which 37 cones were measured (Fig. 5.3). Again, one of these was an S cone and the other 36 fell into two distinct populations of L and M cones, with an L : M ratio of 2 : 1.

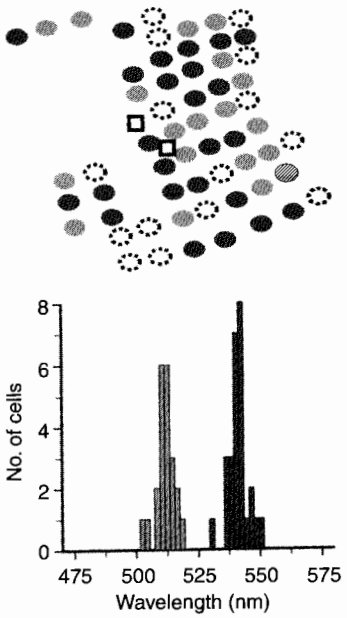

Figure 5.2 Foveal sample from a male (H24). Dark circles, L cones; light circles, M cones; ◍, S cone; dashed circles, cones not possible to measure; open squares, rods out of the plane of the cone array. Histograms of the distribution of the λ_{max} of individual L and M cones. The ratio of L : M cones is 1.2 : 1.

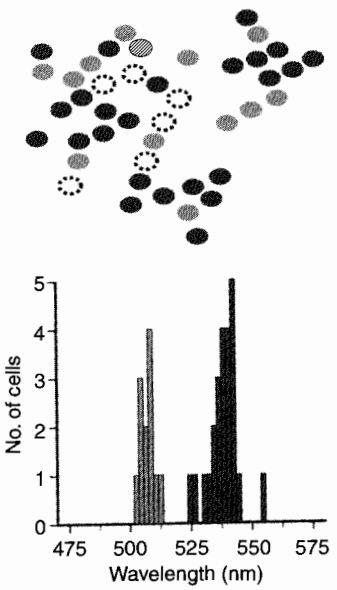

Figure 5.3 Foveal sample from a female (H25). Dark circles, L cones; light circles, M cones; ◍, S cone; dashed circles, cones not possible to measure. Histograms of the distribution of the λ_{max} of individual L and M cones. The ratio of L : M cones is 2.0 : 1.

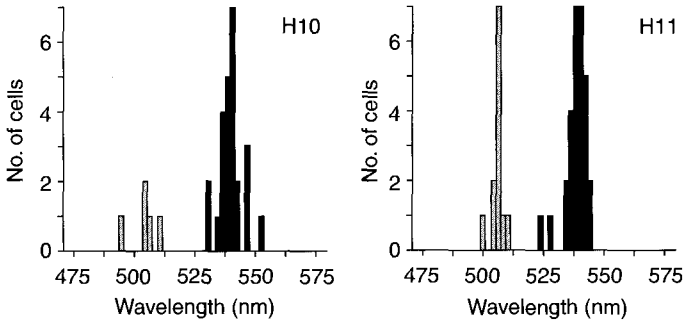

Figure 5.4 Histograms of the distribution of the λ_{max} of individual L (black bars) and M cones (grey bars) from two further males, H10 and H11. The ratio of L : M cones is 5.0 : 1 for H10 and 2.4 : 1 for H11.

The further two retinae were both from males (H10 aged 48 and H11 aged 45) and yielded a sufficiently large number of cones, though not in a clear array, to give ratios of L : M cones. In H10, 30 cones were recorded with an L : M ratio of 5 : 1, whereas in H11, 41 cones gave a distinctly different L : M ratio of 2.4 : 1 (Fig. 5.4).

Human tissue—ora serrata

Tissue from this region proved very difficult to record from, primarily because of excessive vitreous humour adhering to the retina. This proved almost impossible to remove and problems were further exacerbated by unavoidable contamination from pigment epithelium. Only in H24 were we able to make any significant measurements and here we recorded from only nine cones. Nevertheless, eight of the cones were L cones, which is suggestive of a considerably higher ratio of L : M cones than in the fovea.

Marmoset tissue

A relatively large parafoveal array was obtained from an individual female marmoset (Fig. 5.5). A total of 50 cones were recorded and these could be readily divided into two populations with the ratio of the longer to the shorter of 0.7 : 1.

Discussion

Human

The ratio of L : M cones in the four individuals ranged from 1.2 : 1 to 5.0 : 1 (Table 5.1). In total, from the four retinae, 156 L and M cones were identified with a mean ratio of 2.1 : 1. This figure agrees remarkably well with an overall ratio derived from microspectrophotometric data from twelve previous human eyes (some published (Dartnall *et al.* 1983*a*, *b*) and some not) that were obtained directly from enucleations and were not bleached. From these earlier twelve eyes, a total of 450 cones were measured, 303 L and 147 M, giving a ratio of 2.1 : 1. Although the numbers from any single retina were small,

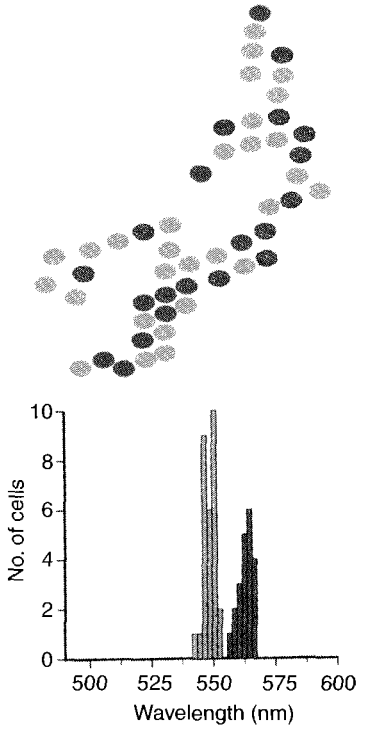

Figure 5.5 Foveal sample from a female marmoset (*Callithrix jacchus jacchus*). Dark circles: longer-wave cones; light circles: shorter-wave cones. Histograms of the distribution of the λ_{max} of individual cones. The ratio of the longer cones to the shorter is 0.7 : 1.

Table 5.1 Human foveal samples

Subject, age (years)	L : M cones	L : M ratio
H24 male, 56	27 : 22	1.23 : 1
H25 female, 48	24 : 12	2.00 : 1
H11 male, 45	29 : 12	2.42 : 1
H10 male, 48	25 : 5	5.00 : 1
Total	105 : 51	2.06 : 1

the individual ratios ranged from 0.9 : 1 to 4.3 : 1, a range not dissimilar from that of the four reconstituted retinae presented in this paper, 1.2 : 1 to 5.0 : 1.

These data strongly suggest that the ratio of L : M cones in the foveal region in humans varies between individuals from close to unity to at least as high as 5L : 1M. However, the average for the population is close to 2 : 1 (Table 5.1).

Although the size of the arrays was small, it is clear that the L and M cones do not form a systematic alternating array (e.g. Fig. 5.2), but appear to be randomly arranged

with some indication of clumping. A χ^2 test in the array from H24 showed that the frequencies of three possible types of transition from cone to cone (L–L, M–M or L–M, in the direction of the long axis of the array), did not differ significantly from chance ($\chi^2 = 2.86$, d.f. $= 2$), supporting a random distribution, but with a bias towards clumping or aggregation.

Comparison with other species

It is clear that in humans the relative proportion of L and M cones is on average about 2 : 1, but the ratio varies between individuals from about 1 : 1 to at least as high as 6 : 1, with some suggestion that the ratio may be even higher in extreme cases. This could be argued to reflect the order of L and M genes and their relative distances from the LCR in the gene array on the X chromosome. The first gene is usually an L gene and the first gene may be most likely to be coupled to the LCR (Winderickx *et al.* 1992; Yamaguchi *et al.* 1997). A weakness of this argument, however, is that the ratio of L to M cones appears to be lower in Old World monkeys and Apes, even though the arrangement of the gene array is thought to be similar across catarrhine primates (Dulai *et al.* 1994, 1999; Onishi *et al.* 1999). Data for Great Apes appears to be limited to the chimpanzee (*Pan troglodytes*), which has an estimated ratio of about 1.3 (Jacobs *et al.* 1996). Our direct microspectrophotometric studies of foveal tissue from ten talapoin monkeys (*Miopithecus talapoin*) gave an overall ratio of 0.9 : 1 (Mollon and Bowmaker 1992) from 545 cones. The individual ratios varied from 0.4 to 1.6. A similar collection of data from a total of 219 cones from macaque monkeys gave a ratio of 0.7 : 1 (unpublished). In addition, data from four baboons (*Papio papio*) (Bowmaker *et al.* 1991) gave a ratio from 120 cones of 0.7 : 1 with a range from 0.5 to 1.1, and individuals from five further catarrhine species gave a mean ratio of 1.2 from a total of 231 cones, with the individual ratios ranging from 0.8 to 2.5 (Bowmaker *et al.* 1991).

Estimates of the proportions of L and M cones in macaques, derived from analyses of the spectral luminosity function (Dobkins *et al.* 2000) were consistent with a cone ratio of 1 : 1; and a ratio of 1.4 : 1 was found from a large array of more than 900 cones in an individual macaque by spatially localized retinal densitometry made possible by adaptive optics (Roorda *et al.* 2001).

This apparent difference between humans and Old World monkeys is further supported by findings from analysis of mRNA in whole retinae (Deeb *et al.* 2000). The L : M ratio of mRNA from 26 monkeys, primarily *Macaca nemestrina*, ranged from 0.6 to 7.0 with a mean of about 1.6. This is markedly different from a similar analysis of mRNA from more than 50 human retinae where the range was from about 1–10 with a mode of 4 (Yamaguchi *et al.* 1997). These L : M ratios, somewhat higher than physiological estimates, may be a consequence of whole retinal extracts that will reflect the overall retinal ratio and not that of the foveal region (Neitz *et al.* 1996; Yamaguchi *et al.* 1997), but nonetheless, they reinforce the evidence for a difference between human and Old World monkeys.

Marmoset

In the case of the single female marmoset the two types of long-wave cone were in the ratio 0.7 : 1. This value does not differ significantly from 1.0 ($\chi^2 = 0.98$, d.f. = 1), the value that would be expected if X-chromosome inactivation were truly random and did not favour the maternal or the paternal chromosome. A ratio of unity similarly gave the simplest description of earlier data for another New World monkey, the squirrel monkey, *Saimiri sciureus* (Bowmaker *et al.* 1985).

A χ^2 analysis of transitions, similar to that performed on the array of H24, shows that the marmoset array does not depart significantly from chance ($\chi^2 = 4.21$, d.f. = 2). It is often held that X-chromosome inactivation occurs at an early embryonic stage, when the number of cells is small, and that the same chromosome remains inactivated in all the descendants of a given cell. The randomness observed in the marmoset photoreceptor matrix suggests either that the specificity of a cone is determined only at the last cell division or that a mixing of retinal cells is ensured by an active migration of cones during development.

Functional significance

What might be the functional significance of the individual variation in the ratio of L and M cones in humans? Traditionally, the ratio is held to affect the photopic luminous efficiency function, as measured by, say, flicker photometry (De Vries 1947); and indeed Rushton and Baker (1964) found an association between flicker photometric settings and reflection densitometric measurements of the proportions of L and M pigments in individual retinas. The relative numbers of L and M cones should not, however, affect Rayleigh equations: when subjects make a foveal colour match, they are thought to equate the quantum catches in the photopigments on the two sides of the stimulus field and so the equation ought to hold for each individual cone, whatever the numbers of a given type of cone. It has been proposed that settings of unique yellow reflect the ratio of L and M cones (Cicerone 1990), but differences in the expected direction are not found between female carriers of protan and deutan deficiencies, who are thought to have low numbers of L and M cones, respectively (Mollon and Jordan 1997; Miyahara *et al.* 1998).

A disproportion in the two types of cone should also affect the spatial properties of chromatic discrimination. Midget ganglion cells, which are thought to carry the L/M opponent signal, draw their centre input from a single cone. According to one hypothesis (Lennie *et al.* 1991), the antagonistic surround is drawn arbitrarily from both L and M cones in the neighbourhood. On this account, since a superabundance of one type of cone will produce homogeneous patches of mosaic, many midget ganglion cells would not be chromatically opponent. However, any ganglion cell drawing its centre input from the minority type of cone would be strongly opponent, since its surround would on average be purer than it would be in a more equilibrated retina. According to the hypothesis of Reid and Shapley (1992), the surround input is drawn specifically from cones of the type opposite to the cone that gives the centre input. On this account,

a ganglion cell drawing its centre input from the superabundant class would have on average to seek its opponent input at a greater distance than would such a cell in an equilibrated retina, but the opposite would be true for a ganglion cell drawing its centre input from the minority type.

Acknowledgement

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