

# Cone pigment gene expression in individual photoreceptors and the chromatic topography of the retina

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Received July 22, 1999; revised manuscript received October 5, 1999; accepted October 5, 1999

Human trichromatic vision is based on three classes of cones: L, M, and S (long-, middle-, and short-wavelength sensitive, respectively). Individuals can have more than one M and/or more than one L pigment gene on the X chromosome along with an S pigment gene on chromosome 7. In some people the X-linked pigment gene array can include polymorphic variants that encode multiple, spectrally distinct cone photopigment subtypes. A single-cell, polymerase chain reaction approach was used to examine visual pigment gene expression in individual human cone cells and identify them as L or M. The ratio of L:M pigment gene expression was assayed in homogenized retinal tissues taken from the same eyes. Results indicate that there is a close correspondence between the cone ratio determined from counting single cells and the L:M pigment mRNA ratio estimated from homogenized pieces of retina. The results also show that the different pigment genes in one array are often expressed at very different levels, giving rise to unequal numbers of L and M cones. Expression of only one photopigment gene was detected in each cone cell. However, individual males can have more than the classically described three spectrally distinct cone types in their retinas. © 2000 Optical Society of America [S0740-3232(00)01503-9]

*OCIS codes:* 330.1720, 330.5310.

## 1. INTRODUCTION

In a sense, trichromacy refers to a limitation of color vision—a trichromat can be deceived—in which simple mixtures of three primary colors can exactly mimic complex compositions of wavelengths reflected from natural objects. In our modern technological world the deficiencies inherent in trichromacy can be exploited to advantage. For example, for the trichromatic eye, color reproduction is enormously simplified. Trichromats accept mixtures of three colors, such as are seen from color television and computer screens, as reasonable representations of the real, more complicated spectral world. Such simple devices for representing color images would not be possible if our eyes were perfect point-by-point spectrum analyzers.

Two centuries ago Thomas Young suggested that the number of receptors or “particles” in the eye is what limits human color vision to trichromacy.<sup>1</sup> He recognized that an ideal spectrum analyzer would have a large number of separate receptor types to sample across all visible wavelengths independently, but, in contrast, for human vision the number of independent receptors must be limited by constraints inherent in the biology of the eye. A modern version of the Young–Helmholtz theory of trichromacy holds that the number of cone photoreceptor types is confined to three, each filled with a different photopigment, abbreviated L, M, and S (long-, middle- and short-wavelength sensitive, respectively). For many years, it was expected, as a logical extension of this classical theory, that three genes would encode the protein components of the corresponding three pigments. Inheritance patterns of color vision defects suggested that the

M and L photopigment genes were on the X chromosome and the S pigment gene was on an autosome.

For more than a dozen years it has been known that, contrary to expectation, there is variability in the number of cone pigment genes on the X chromosome.<sup>2</sup> A rich variety of pigment gene arrangements occur in the population, which can include multiple M and multiple L photopigment genes in a tandemly repeated array.<sup>3–7</sup> Another shift away from classical thinking over the past decade has been the realization that there is variation in the absorption spectrum within the L and M classes of pigments. Amino acid polymorphisms produce spectral differences in absorption properties among the pigments so that in the normal human population there are subtypes of M and subtypes of L pigments.<sup>4,8–10</sup> These normal individual differences in photopigments are known to play a role in normal variation in color vision.<sup>9,10</sup>

Finally, genes that encode different photopigment subtypes can occur on the X chromosome of an individual male to provide him with the genetic potential to have more than three spectrally distinct cone pigments. The presence of extra pigment genes and variations in the numbers, ratios, types, and subtypes of pigments encoded on the X chromosome complicates understanding of the relationship between the pigment genes and the chromatic topography of the retina.

Expression of the extra pigment genes has been investigated through the examination of photopigment messenger RNA's (mRNA's) from retinal homogenates derived from human donor eyes. Although the results indicate that many people may have one or more photopigment genes in their X-chromosome arrays that are not

detectably expressed,<sup>6,7,11,12</sup> from a screening of more than 100 human eyes it was found that more than two genes from the X-chromosome array are expressed in some men.<sup>13</sup> Current information about expression of the X-encoded cone pigments can be summarized as follows. The individual photopigment genes within a person's array often differ in their levels of expression.<sup>13</sup> Usually, mRNA from one gene, an L pigment gene that is positioned first in the array, is expressed at a higher level than all the other M and L pigment genes combined.<sup>13</sup> M photopigment genes positioned downstream in the array are usually expressed at a lower level than the L gene first in the array.<sup>14</sup> However, there do appear to be exceptions: in some people, downstream M genes are expressed at equal or even at slightly higher levels than the L gene first in the array. Approximately 8% of men appear to express a second L pigment gene subtype at a lower but still significant level (greater than 20% of the level of the predominant L) in addition to M and S pigment genes.<sup>13</sup> Thus men can express two subtypes of L pigment mRNA in addition to M and S pigment mRNA, specifying a total of more than three spectrally unique pigments.

Experiments that have examined cone pigment mRNA from retinal homogenates provide information about photopigment gene expression averaged over a large collection of cones. However, many questions are left unanswered about the distribution of photopigments within the individual cones. To address these questions we have developed methods that employ the polymerase chain reaction (PCR) to examine the photopigment mRNA contents of individual cone cells. One question that has been addressed with this approach is whether the expression of the extra genes can produce separate subtypes of cones in one eye.<sup>15,16</sup> In experiments in which pigment expression was examined in 110 individual cones from one eye of a male eye donor, we found evidence that the extra genes can lead to additional cone subtypes. The detailed results and analysis regarding the presence of more than three cone types in his eye will be presented in a forthcoming report. However, in summary, one L pigment subtype was expressed in the majority of the cones of this eye donor. He also had a population of M cones. In addition to the major populations of M and L cones, there was a small but distinct subpopulation of cones that expressed a second L pigment subtype. The results are consistent with the idea that each cell that is destined to become an L or M cone "chooses" to express one gene from the X-chromosome pigment gene array. A given cone is most likely to choose to express the first gene in the array, which encodes an L pigment in males with normal color vision. Usually, fewer cones choose to express any one of the distal genes. In some individual males, such as in one eye donor that we examined, genes downstream of the first two in the array can be transcribed to produce additional distinct subpopulations of cones in sufficient numbers to be significant for vision.

In the experiments reported here we analyze the mRNA contents of individual human cones to address two issues relevant to the chromatic topography of the retina: (1) We examine the relationship between L:M cone ratios and the relative L:M mRNA levels in retinal homoge-

nates, and (2) we evaluate the assumption that each cone expresses only one photopigment gene from the X-chromosome array. The examination of relative amounts of cone pigment mRNA's from homogenates of whole retinas and retinal patches has emerged as a unique tool for gaining information about the chromatic topography of the human retina. The technique has been used to examine retinas from people presumed to have had normal color vision<sup>6,13,14,17,18</sup> and retinas from people presumed to have color vision defects.<sup>7,12</sup> In those mRNA studies it has sometimes been assumed that the relative mRNA levels reflect the relative numbers of cones. The experiments reported here provide evidence that the assumption is valid and that each cone expresses only one pigment gene from the X-chromosome array.

## 2. METHODS

### A. Donor Eyes

Human eyes were obtained through the Wisconsin Lions Eye Bank. Eyes were enucleated and refrigerated within 3 h of donor death. Eyes from four Caucasian male donors aged 5, 6, 43, and 62 years were examined (see Table 3 below).

### B. Retinal Dissection and Single Cone Isolation

Anterior segments were removed, and the posterior poles were submerged for several hours in cold Dulbecco's modified Eagles medium supplemented with 10% sucrose. Subsequently, the retina was removed from each eyecup, placed in a petri dish, and submerged in culture medium.

*Single-Cell Isolation.* In one eye, a trephine was used to punch a 6-mm patch of retina centered on the fovea. The outside edge of this patch corresponded to a visual angle of approximately 10° eccentric from the fovea. Single cones were collected from retinal regions between 3 and 6 mm away from the center of the macula, which corresponds to visual angles in the living eye from approximately 10° to 20° eccentric from the fovea. Once the central patch of tissue was removed, the photoreceptor layer was teased off the retina at the edge cut by the trephine and at the optic nerve head tear. The pieces of tissue were collected along with ~100  $\mu$ l of culture medium and placed into a tube on ice. The tissue was gently disrupted by trituration in and out of a pipette tip, and transferred to the well of a microscope slide that contained culture medium. At this point the fragments of the photoreceptor mosaic existed as rafts of several hundred cells. Within a few minutes the photoreceptor mosaic began to self-dissociate, and individual cone cells were free in the medium. Single cone cells were collected by reverse pressure in a 10–15- $\mu$ m micropipette guided under observation through a microscope with Hoffman contrast optics. Each individual cone cell was released along with approximately 50–150 nl of culture medium onto the inside sidewall of a 0.5-ml Perkin–Elmer thin-wall PCR tube. One  $\mu$ l of TE (10 mM tris-HCL, 1 mM ethylenediamine tetra-acetic acid, pH 8.0) was placed on top of the cell. The tube was centrifuged to drive the

drop of TE and the cell to the bottom of the tube; it was then immediately placed in a dry ice/ethanol bath and stored at  $-80^{\circ}\text{C}$ .

In the fellow eye, a 6-mm patch of retina was isolated from the same retinal eccentricity as the collected single cone cells. This retinal patch was stored at  $-80^{\circ}\text{C}$  and was used in an experiment separate from yet parallel to the single-cell analysis.

### C. RNA Isolation and Reverse Transcription

*Single Photoreceptor Cells.* Five  $\mu\text{l}$  of GeneReleaser (BioVentures) was added to each cell inside the original collection tube. The cell/GeneReleaser mixture was vortexed for 30 s and then microwaved for 3 min at 900 W. The reaction components required for reverse transcription (RT) of RNA into cDNA were added to the tube. The final reaction volume was 25  $\mu\text{l}$ . RT was done with the RNA-PCR kit from Perkin-Elmer according to the manufacturer's protocol. Cycling parameters were  $23^{\circ}\text{C}$  for 10 min,  $42^{\circ}\text{C}$  for 15 min,  $99^{\circ}\text{C}$  for 5 min, and  $5^{\circ}\text{C}$  for 5 min.

*Tissue Patches.* Nucleic acids were isolated from retinal tissue patches as described previously.<sup>18</sup> One  $\mu\text{l}$  of nucleic acid in TE was reverse transcribed as described for single photoreceptor cells except that the final reaction volume was 20  $\mu\text{l}$ .

### D. Polymerase Chain Reaction

*Single Photoreceptor Cells.* Aliquots of the RT reaction were used in the PCR. Primers were designed so the

PCR product from genomic DNA versus cDNA could be distinguished by their sizes. Primer sequences are described in Table 1, and the thermal cycling parameters for each primer pair are described in Table 2. Hot-start PCR was done with use of the XL-PCR kit and AmpliWax Gem 100's from Perkin-Elmer as described by the manufacturer. Optimal reaction conditions required 1.4 mM  $\text{Mg}^{2+}$  and 30 pmol of each primer in a final reaction volume of 100  $\mu\text{l}$ .

A flow diagram of the single-cell collection and RNA-PCR amplification procedure is illustrated in Fig. 1. The general strategy used to amplify fragments of the visual pigment cDNA obtained from each cell is outlined in Fig. 2(A). First, one half of the RT reaction was used in PCR with primers 2 and 7 to amplify a segment of both L and M pigment cDNA's extending from the 5' end of exon 3 to the middle of exon 5, including the sequences that encoded the two amino acid positions that determine whether the specified pigment will be L or M. Primers 2 and 7 hybridize to regions of DNA that are identical for L and M photopigment genes and thus will amplify M or L cDNA's but not S cDNA. For two of the four donors, when no PCR product was obtained with primers 2 and 7, the remaining half of the RT reaction was used in PCR with primers 8 and 9 specific to S pigment cDNA.

Amplified M and L pigment cDNA's were gel purified, and 1  $\mu\text{l}$  was used with primers 5 and 7 to amplify a segment extending from the 3' end of exon 4 to the middle of exon 5. The product was used in an RsaI restriction en-

**Table 1. Primers Used in the PCR**

Primer	Nucleotide Position <sup>a</sup>	Specificity	Exon	Sequence
1	904-924 (F)	L and M	3	5'GGATCACAGGTCTCTGGTCTC3'
2	904-927 (F)	L and M	3	5'GGATCACAGGTCTCTGGTCTCTGG3'
3	1053-1037 (R)	L and M	3	5'GCGGGGCTGTCCACACA3'
4	1167-1146 (R)	L and M	4	5'CCATGAGGACAATCATGTAAGA3'
5	1232-1250 (F)	L and M	4	5'CCGAGCGGTGGCAAAGCAG3'
6	1328-1307 (R)	L	5	5'GCAGTACGCAAAGATCATCACC3'
7	1374-1356 (R)	L and M	5	5'TGGCAGCAGCAAAGCATGC3'
8	581-601 (F)	S	1	5'GCCACACTGCGCTACAAAAG3'
9	1037-1014 (R)	S	3	5'TGAAGCAGAAGATGAAGAGGAACC3'

<sup>a</sup>Nucleotide numbering of Ref. 2. In parentheses, F or R indicates the direction of the primer as forward or reverse, respectively.

**Table 2. PCR Thermal Cycling Parameters**

Primer Pair	Genes <sup>a</sup> Amplified	Initial Cycle	Cycling Parameters	Number of Cycles	Final Cycle	Experiment
2 and 7	L and M	94C 5 min	94C 1 min, 59C 1 min, 72C 1 min 94C 30 s, 59C 30 s, 72C 45 s	10 25	72C 10 min	Single Cells
1 and 4	L and M	94C 5 min	94C 45 s, 57C 45 s, 72C 45 s	35	72C 10 min	Single Cells
5 and 7	L and M	94C 5 min	70C 1 min, 94C 1 min	35	72C 10 min	Single Cells
8 and 9	S	94C 5 min	94C 1 min, 59C 1 min, 72C 1 min 94C 30 s, 59C 30 s, 72C 45 s	10 25	72C 10 min	Single Cells
5 and 7	L and M	94C 5 min	94C 45 s, 57C 45 s, 72C 45 s	40	72C 10 min	Retinal Patches
1 and 6	L	94C 5 min	94C 45 s, 61C 45 s, 72C 45 s	40	72C 10 min	Retinal Patches
1 and 4	L and M	94C 5 min	94C 45 s, 57C 45 s, 72C 45 s	40	72C 10 min	Retinal Patches

<sup>a</sup>The short form L and M indicates that the primer pair amplifies a segment from both L and M genes, L indicates that it amplifies a segment from only the L gene, and S indicates that it amplifies a segment from only the S gene.

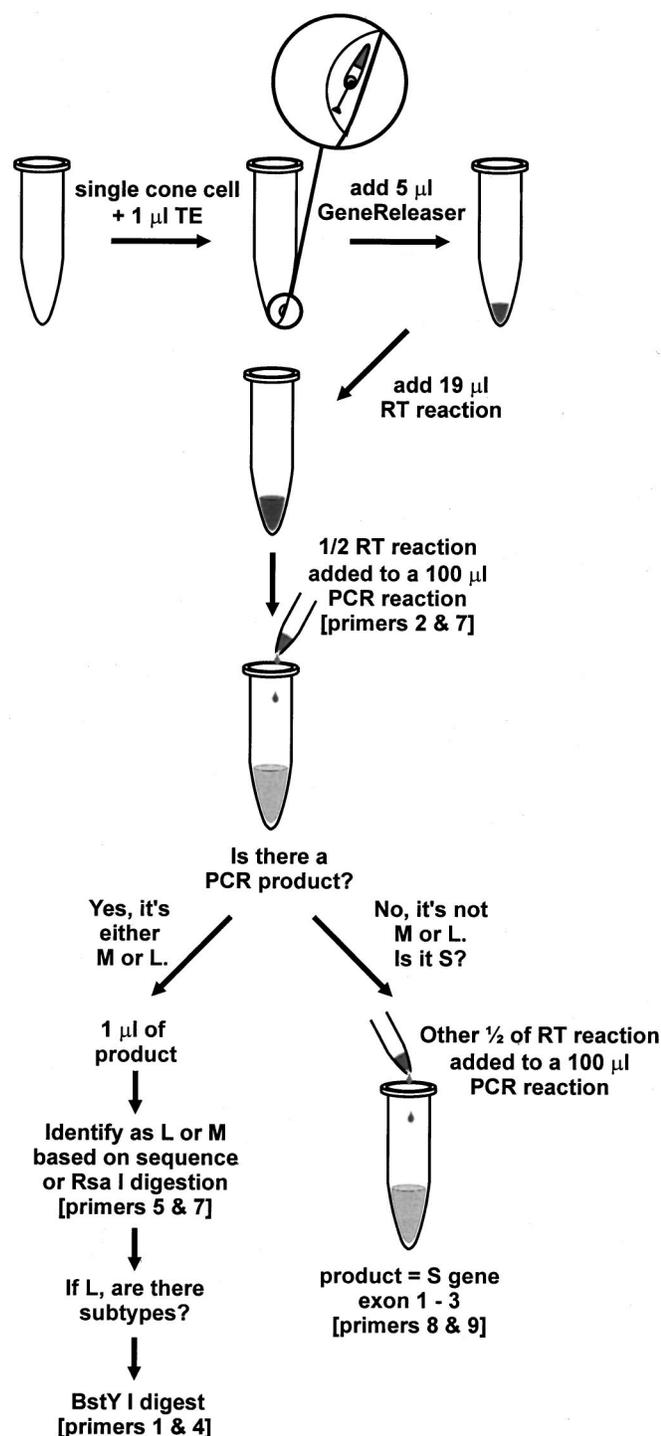


Fig. 1. Illustration showing each step of the pathway a single cone cell travels during the isolation and amplification procedure.

zyme digest. The DNA restriction enzyme, endonuclease *RsaI*, cleaves within exon 5 of L but not M genes (see map at the top of Fig. 2). Cells identified as L cones with this assay were subjected to further analysis. One  $\mu\text{l}$  of the cDNA product amplified by primers 2 and 7 (above) was used in PCR to amplify a region extending from the 5' end of exon 3 to the 5' end of exon 4. This was done with pairing primers 1 and 4. PCR products were used in a restriction digest assay with endonuclease *BstYI*. This

restriction enzyme either cleaves exon 3 or does not, depending on the nucleotide present at position 1026 (see map at the top of Fig. 2). The *BstYI* restriction assay was used to identify two subtypes of the L photopigment gene that differ at position 1026. The *BstYI* restriction assay was also used in experiments designed to determine whether only a single subtype of L pigment is expressed in each cell in the case of a retina in which more than one L subtype was expressed.

**Tissue Patches.** An 8- $\mu\text{l}$  aliquot of the visual pigment cDNA was amplified by hot-start PCR with AmpliWax PCR Gem 50's from Perkin-Elmer. Optimized PCR reactions contained 1.5 mM  $\text{Mg}^{2+}$  and 20 pmols of each primer in a final reaction volume of 50  $\mu\text{l}$ . The strategy for PCR amplification of L and M pigment cDNA from retinal patches is illustrated in Fig. 2(B).

To determine the ratio of L:M pigment cDNA's in each retina, we amplified a segment of the L and M pigment cDNA's extending from the 3' end of exon 4 to the middle of exon 5 with use of primers 7 and 9. The product was used in an *RsaI* restriction enzyme digest as described for the single-cell analysis (above). To determine whether more than one subtype of L pigment was expressed in individual retinas, an aliquot of reverse transcribed retinal RNA was used in PCR with primers 2 and 8. These primers specifically amplify a segment of L pigment cDNA extending from exon 3 into exon 5. The selectivity of the L-gene-specific exon 5 primer was demonstrated previously.<sup>14</sup> L pigment cDNA's were gel purified. One  $\mu\text{l}$  was used in a second round of PCR to amplify L-specific exon 3. The PCR product obtained from primers 2 and 6 was used in a *BstYI* restriction assay as described above for single cells. We monitored all PCR experiments for contamination of reactions by carrying several no-template control reactions through each experiment.

#### E. Restriction Endonuclease Analysis and Sequence Analysis

PCR products from single photoreceptor cells and tissue patches were analyzed either by PhosphorImage analysis or by FluorImage analysis. In the PhosphorImage method, products were purified by ultrafiltration, end labeled with  $^{32}\text{P}$ , and digested with either *RsaI*, or *BstYI*. Digestion products were electrophoretically separated on a polyacrylamide gel; the gels were fixed and dried onto filter paper. The cDNA was imaged with PhosphorImager (Molecular Dynamics), and the amount of radioactivity in each band was quantitated by ImageQuant software (Molecular Dynamics). In the fluorescence method, PCR products were purified by ultrafiltration and directly incubated with the appropriate enzyme. Digestion products were electrophoretically separated on a polyacrylamide gel. The gels were fixed and incubated in a solution of 0.5- $\mu\text{g}/\text{ml}$  ethidium bromide for 20 min followed by 20 min of destaining in water. The amount of DNA in each band was estimated from quantitation of the fluorescence signal with the FluorImager SI (Molecular Dynamics). The validity of the quantitative analysis with the FluorImager was first established by comparing the FluorIm-

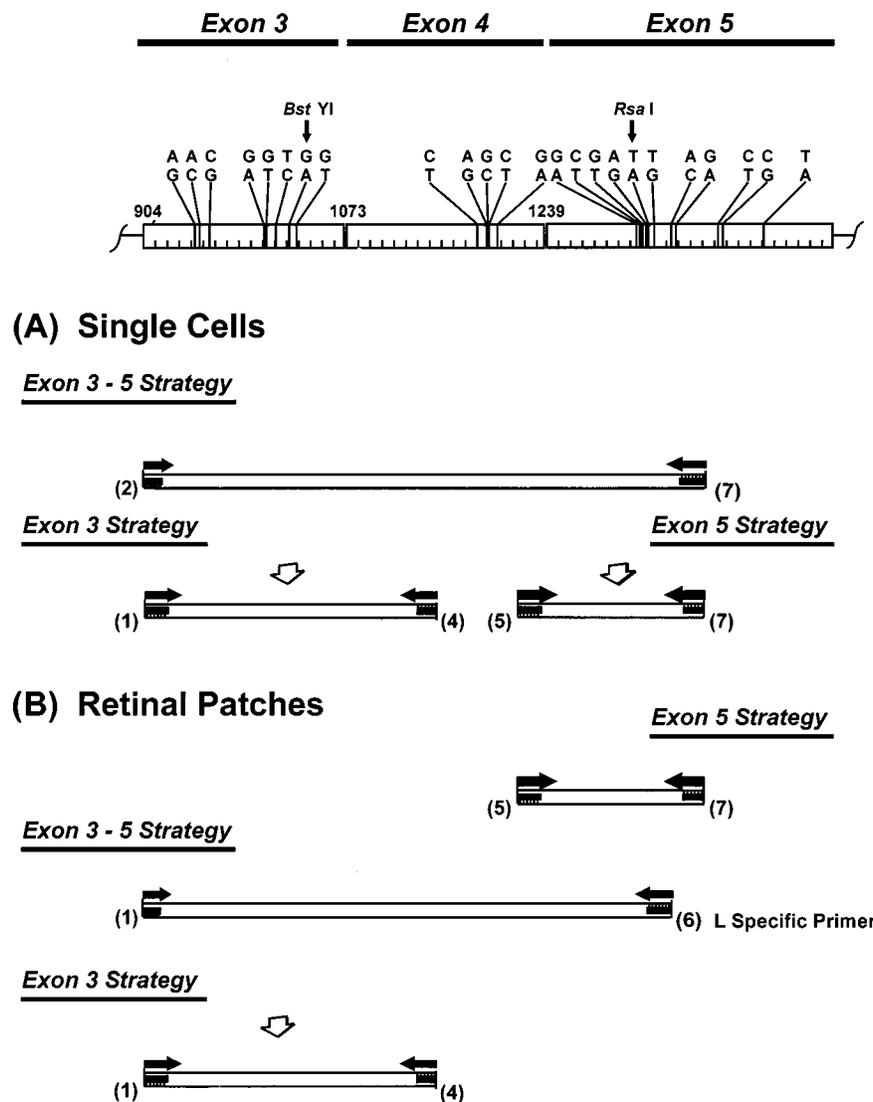


Fig. 2. (A) Strategy for PCR amplification from single cone cells. L or M cDNA fragments containing exons 3–5, exon 3, and exon 5 were amplified. The number labels of the primers correspond to those in Table 1, where the primer sequences and their specificities are described. (B) Strategy for PCR amplification from retinal patches. L and M cDNA fragments containing a portion of exon 5 were amplified for use in experiments to estimate M/L cone ratio. A two-step process amplified L-specific exon 3 fragments. The L cDNA exon 3–5 PCR product was used in a second round of PCR to amplify L-specific exon 3.

aging results with those obtained by PhosphorImaging. The two methods gave similar results for individual samples.

cDNA's were sequenced from several single cells to verify restriction digestion analysis with methods previously described.<sup>4</sup>

### 3. RESULTS

Figure 3(A) shows the pieces of photoreceptor mosaic viewed under a microscope with Hoffman contrast optics. The rods are packed tightly and form a dark matrix. Individual cones are located in larger light spaces. At this eccentricity the cones have very large diameters compared with the rods and constitute ~5% of photoreceptors. They are nearly transparent when viewed axially except for the small outer segment, which can be seen as a dark dot in the space occupied by the cone. Within a few minutes, the photoreceptor mosaic began to self-dissociate [Fig. 3(B)] and morphologically distinguishable

rods and cones were visible, as shown in Fig. 4(A). Single identifiable cone cells were collected by reverse pressure in a 10–15- $\mu$ m micropipette guided under observation [Fig. 4(A)]. Each individual cone cell was released into a collection tube under examination along with approximately 50–150 nl culture medium fluid collected with the cell. The amount of fluid was just barely visible to the naked eye on the side of the collection tube. Expulsion was guided under microscope observation through the semitransparent wall of the tube. A fraction of the cells could be seen as they were expelled into the tube, verifying single-cell isolation as shown in Fig. 4(B). The frozen single cells were stored in their tubes at  $-80^{\circ}\text{C}$  until after the analysis of tissue patches from the fellow eye was completed.

#### A. Proportion of M to L mRNA

For each of the four individuals from whom we collected single cone cells, we assayed for the presence of L and M

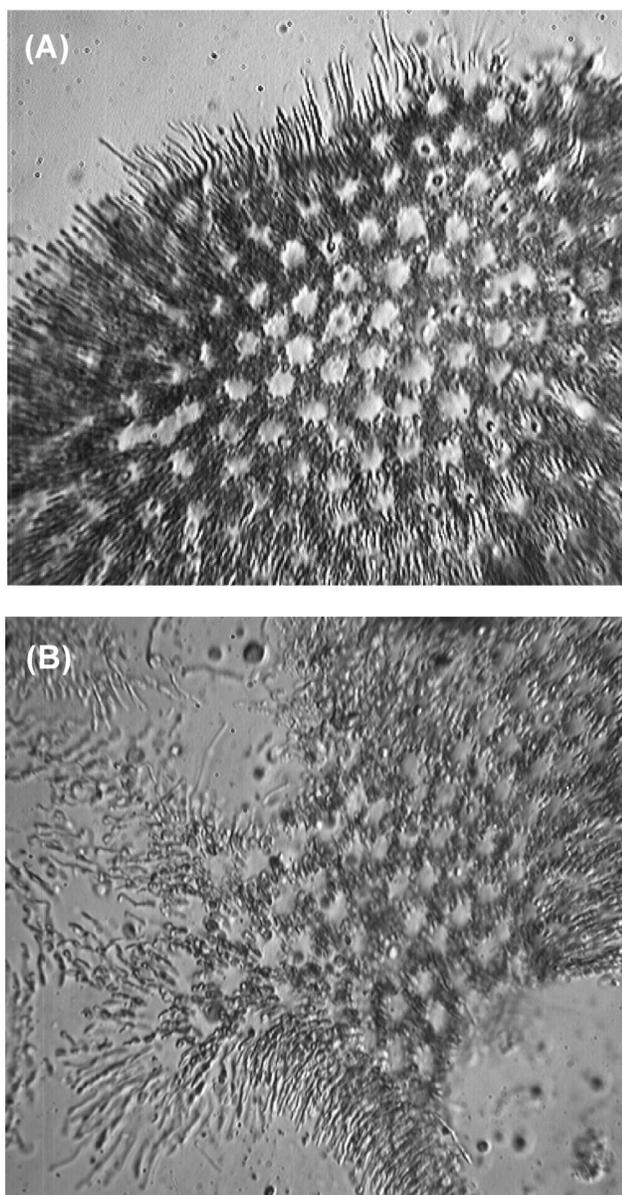


Fig. 3. (A) Patch of human photoreceptor mosaic obtained from a male eye donor. The image is magnified  $2000\times$  under Hoffman contrast optics. Rods are packed tightly, individual cones are transparent in this view and are located in the larger spaces separated by rods. (B) Patch of human photoreceptor beginning to self-dissociate. Planks of rods and cones are seen breaking away and dispersing from the intact mosaic.

cone pigment mRNA's. The assay was performed on the retinal tissue patch isolated from the fellow eye at the same retinal eccentricity as that of the collected single cells. The results are shown in Fig. 5. The autoradiograms were digitally imaged and quantitated by Phosphor Image analysis, as previously described.<sup>3</sup> All four men expressed more L than M pigment mRNA. This appears to be characteristic of color normal males.<sup>6,14</sup> The estimated proportions of M pigment mRNA calculated as  $M/(M + L)$  are shown in Table 3. The numbers represent the average of at least three replicated measurements; the 95% confidence intervals associated with the mean proportions are given in column 4 of Table 3.

### B. Counting the Number of M and L Cones

A first set of single-cell experiments was designed to determine the relative numbers of M and L cones. Figure 6 shows the results of the quantitative *RsaI* restriction digestion assay performed on five single cones from one subject. Each lane represents the analysis of a single cell. Expression of L or M in each cone was determined from the banding pattern. Two bands are produced from L gene fragments, which are cut by the enzyme. Only one band is produced from an M gene fragment, which is not cut by the enzyme.

The results from the restriction analysis were confirmed for several of the cells by direct sequence analysis. Figure 7 shows a region of exon 5 sequence obtained from cDNA from single cells. The sequences were obtained from two different single cone cells from one individual. The sequences are of fairly high quality considering that the material used originated from one cell. The L sequence in Fig. 7 contains two "strong stops," where bands are seen in three or four lanes. In spite of these artifacts, the nucleotide differences that distinguish M from L in exon 5 can be clearly read.

Table 4 shows the results of single-cone-cell analysis from the four male eye donors. In total, 351 individual cones were collected. Amplification was successful with use of the L and M primer pair for 238 of these cells. For

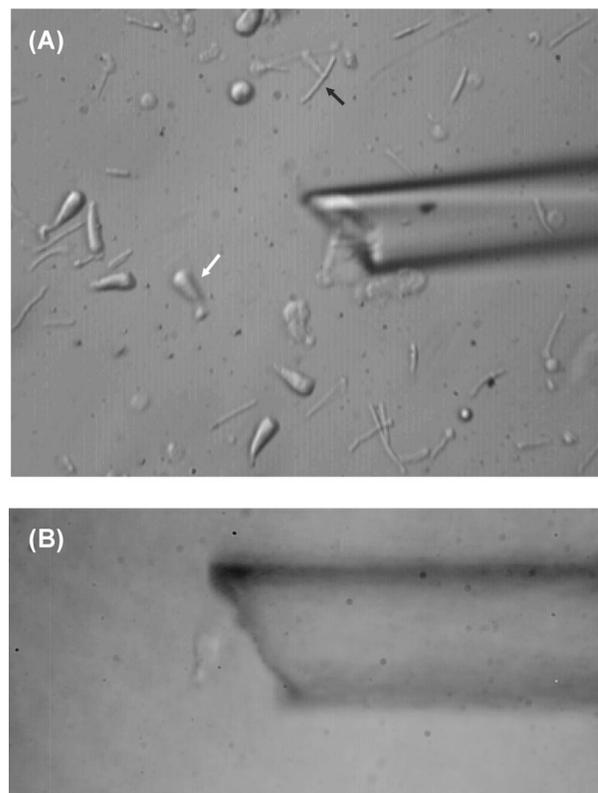


Fig. 4. (A) Typical field of photoreceptor cells. Individual morphologically distinguishable rods (black arrow) and cones (white arrow) are visible. A  $10\text{--}15\ \mu\text{m}$  micropipette guided under observation is used to collect single cones. (B) Individual cone cell alongside the micropipette. The cell was expelled into a 0.5-ml microcentrifuge tube. The image is taken through the wall of the polypropylene tube and magnified  $2000\times$  under Hoffman contrast optics to verify single-cell isolation.

donors 46 and 54, the second half of the RT products from all cells that did not work with the L/M primer were used in PCR with the S cone primer pair. In total, for those two subjects, 227 cells were collected. Amplification was obtained from 178 cells total (64%) from the two donors. Forty-nine cells did not work with either primer pair. For these cells we assume that the mRNA was lost or inaccessible for PCR amplification. We think that the most common cause of this problem was that the cell adhered

to the side of the PCR tube rather than being centrifuged down in the early step of cell collection; however, the cell or its contents could have been lost at any stage in the process after it was collected. In almost all cases for single cells, we observed amplification of only one strongly dominant cDNA species or there was no amplification product; these results for the single cells serve to illustrate the complete lack of contamination present in our PCR assays.

In total from the four donors, 256 cone photoreceptors were analyzed. The L and M cones totaled 238 cells of which 192 were L, an average of 81%, and 46 were M, an average of 19%. Table 3 compares the results from counting single cells with those for the mRNA ratio from tissue patches. The 95% confidence intervals are also shown. There is a close correspondence between the relative proportion of L to M pigment mRNA and the relative proportion of L to M cones in the retina. The percent difference between the two estimates ranged from 1 to 5%.

For subject 46, 9 out of 68 cones were S cones; for subject 54, 9 out of 110 were S cones. The numbers of S cones for subjects 46 and 54 were not statistically significantly different ( $p = 0.31$ ). Combining the S cone results for the two donors, 10% of the cones were S with 95% confidence limits of 6–15%. This percentage is consistent with the percentage of human S cones determined from immunohistochemistry.<sup>19</sup> The assay for S cones was not done for the other two donors, subjects 04 and 63.

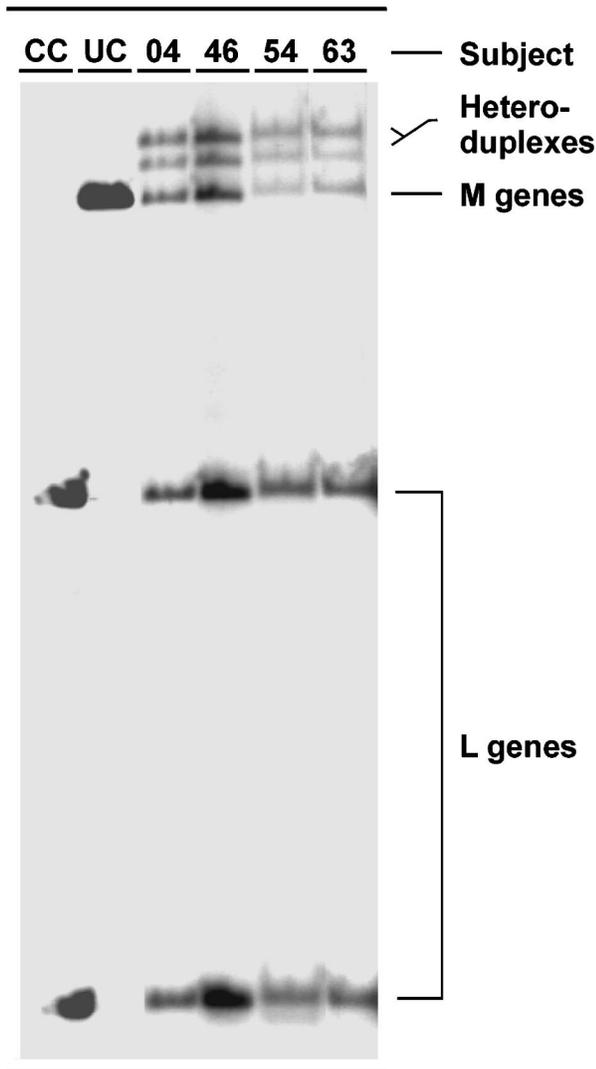


Fig. 5. PhosphorImage analysis of the ratio of M:L cDNA's in the retina of four individuals. Amplified cDNA containing exon 5 was digested with *Rsa*I. It is apparent with the unaided eye that each individual is expressing more L cDNA than M cDNA. Both cut (CC) and uncut (UC) controls are labeled.

**C. Contents of an Individual Cell**

256 individual cone cells were analyzed by a quantitative restriction digestion assay. PhosphorImage analysis provides a precise method for quantitation of the relative amounts of mRNA species within a cell. However, it is important to consider the limitations of the method. In the technique of isolating and collecting a single cone, it is impossible to always avoid collecting a minute amount of nucleic acid from a surrounding lysed cell. This type of contamination would appear in the analysis as two different species, one major and one minor, in one lane on the gel. For example, in Fig. 6 there is an extremely faint band of uncut cDNA in the first L cone lane. This could be due to inefficient cutting by the restriction enzyme. However, the background of uncut cDNA might also be amplification of trace mRNA that leaked from lysed cells that could have been collected in the 50–150 nl of culture medium along with the single cell. In general, we cannot distinguish a trace expression of a second gene from this type of trace contamination.

**Table 3. Comparison of the Proportion of M to L mRNA and M to L Cones**

Subject	Age	Proportion M/L RNA	95% Confidence Interval	Number of Cells Collected	Number of Cells Analyzed	Proportion M/L Cells	95% Confidence Interval
04	43	0.18	0.03–0.32	52	41	0.17	0.07–0.32
46	6	0.15	0.08–0.21	77	68	0.20	0.11–0.33
54	5	0.15	0.04–0.26	150	110	0.18	0.11–0.27
63	62	0.23	0.05–0.40	72	37	0.22	0.10–0.38

As described, *Rsa*I restriction digestion analysis was used to determine an M versus an L cone. Table 5 shows the quantitation results of ten cone cells, five of which

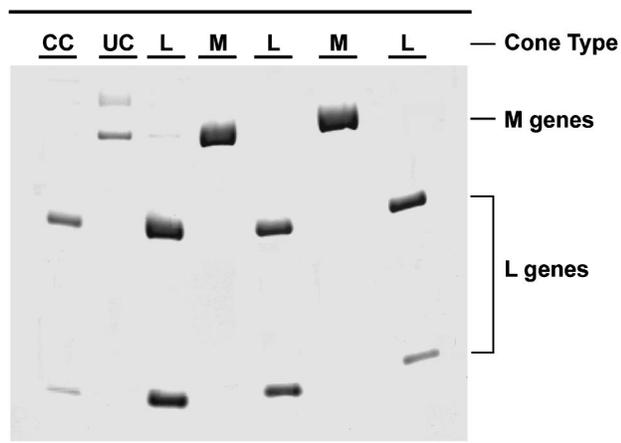


Fig. 6. PhosphorImage analysis of five single cones from one individual. Amplified cDNA containing exon 5 from the X-linked pigment genes was digested with *Rsa*I. Each lane represents the analysis of one cone cell. Three cones expressed an L pigment gene, and two cones expressed an M pigment gene.

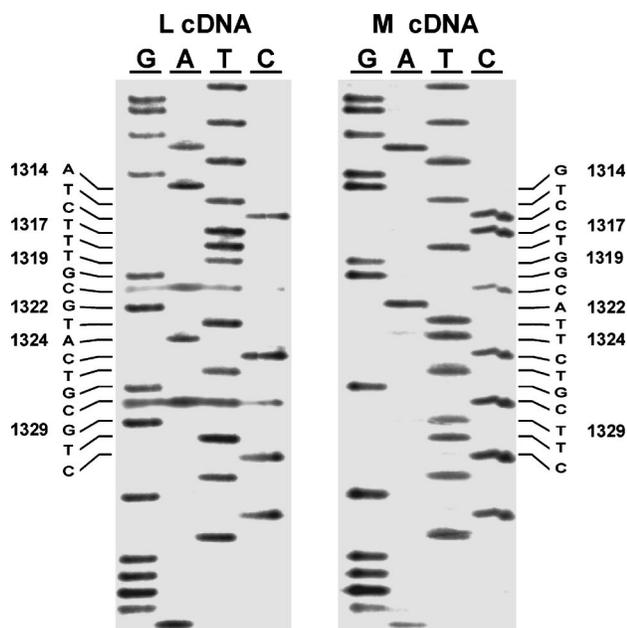


Fig. 7. A 35-base-pair region of exon 5 sequence from two different single cone cells from one individual. One cell contains a cDNA sequence identical to that of an L gene, and the other contains a cDNA sequence identical to that of an M gene. Nucleotide differences that distinguish an L cone from an M cone are labeled.

were M and five of which were L. These were chosen from among the cells that yielded the cleanest results and thus give a best-case indication of how much L is expressed in an M cone and vice versa. Both the uncut and the cut DNA locations on the gel were quantitated for each cell. Cut and uncut controls are also shown, providing a measure of the cutting efficiency of the enzyme and of general background noise on the PhosphorImage. In the cut control, the *Rsa*I enzyme cut with greater than 99% efficiency. All five cells identified as L cones had greater than 99% cut L cDNA. The five cells identified as M cones had an average intensity in the location where the cut bands would be of approximately 0.5% of the uncut cDNA. In each case, the results for the single cells were not different from control samples that contained pure amounts of a single species of cloned cDNA. We conclude from this that expression of a photopigment in a single cone is exclusive to a very high level—greater than 1000 to 1. There is no evidence from our results that there is any M pigment expressed in an L cone or any L pigment expressed in an M cone.

#### D. Exclusivity of Expression of L Cone Subtypes

The mRNA analysis from retinal homogenates showed that subject 54 expressed more than one subtype of L pigment mRNA. In these studies, L cones from subject 54, identified by the exon 5 *Rsa*I restriction analysis, were analyzed further to determine the subtype of L pigment expressed. A restriction-site polymorphism created by nucleotide differences in exon 3 was exploited. For each L cone, the cDNA fragment containing exon 3 of the pigment gene was amplified and cleaved with *Bst*YI. The results showed that, of those cells expressing L genes, 66 (81% of the L cones) expressed one type of L mRNA. This predominant L matched the sequence of the L pigment gene positioned first (most upstream) in the array. However, 15% of the L cones expressed an alternate L cone pigment mRNA. Figure 8 shows the results of the *Bst*YI restriction analysis for five L cones for subject 54. Each lane represents the analysis of a single cone cell. Two bands are seen if one form of an L gene is expressed, and one uncut band is seen if a second form of an L gene is expressed. In this sample of five L cones, three were expressing one form of the L gene at position 1026, and two were expressing an alternative subtype. Results from the *Bst*YI restriction analyses were confirmed by sequence analysis.

An analysis similar to that done for the L and M cones was done to examine exclusivity of expression of the L cone subtypes. We used the *Bst*YI restriction site dimor-

Table 4. Single-Cone-Cell Analysis for Four Male Eye Donors

Subject	Age	Number of Cells					% L	% M	% S
		Collected	Analyzed	L	M	S			
04	43	52	41	34	7	ND	83	17	ND
46	6	77	68	47	12	9	69	18	13
54	5	150	110	82	19	9	75	17	8
63	62	72	37	29	8	ND	78	22	ND
Totals:		351	256	192	46				

**Table 5. PhosphorImage Analysis of Single Cone Cells Digested with RsaI**

Cell Number	Uncut	Cut 1	Cut 2	Uncut/Cut	Proportion Uncut	Cone Type
1	$5.73 \times 10^3$	$1.11 \times 10^6$	$6.51 \times 10^3$	0.0051	0.0051	L
2	$1.87 \times 10^4$	$1.02 \times 10^6$	$1.19 \times 10^6$	0.0084	0.0084	L
3	$7.74 \times 10^2$	$4.46 \times 10^4$	$4.28 \times 10^4$	0.0084	0.0084	L
4	$1.24 \times 10^2$	$3.09 \times 10^4$	$4.53 \times 10^4$	0.0016	0.0016	L
5	$2.19 \times 10^2$	$2.41 \times 10^4$	$3.66 \times 10^4$	0.0036	0.0036	L
Cut Control	$7.29 \times 10^3$	$9.15 \times 10^5$	$7.29 \times 10^5$	0.0044	0.0044	
6	$1.75 \times 10^5$	$5.48 \times 10^2$	$4.31 \times 10^2$	179.17	0.9945	M
7	$1.11 \times 10^6$	$3.77 \times 10^2$	$1.89 \times 10^2$	201.87	0.9951	M
8	$2.63 \times 10^5$	$7.37 \times 10^2$	$3.81 \times 10^2$	235.20	0.9958	M
9	$5.03 \times 10^4$	$3.75 \times 10^2$	$5.33 \times 10^2$	55.40	0.9823	M
10	$3.87 \times 10^4$	$5.90 \times 10^1$	$3.95 \times 10^1$	392.84	0.9975	M
Uncut Control	$1.95 \times 10^4$	$5.32 \times 10^1$	$3.40 \times 10^1$	223.18	0.9955	

phism in exon 3 to examine the subtypes. Table 6 shows the results of ten cells. We chose five cells that express the uncut form of the L gene and five cells that express the cut form of the L gene. Both the uncut and the cut DNA locations on the gel were quantitated for each cell along with cut and uncut controls. The BstYI enzyme cut with greater than 99% efficiency. For the L1 (cut) subtype of cones less than 0.5% of the radioactive signal in the location of the PhosphorImage corresponded to uncut cDNA. The L2 (uncut) subtype of cones had between 99.0% and 99.8% of the signal in the uncut cDNA band. In each case, the values obtained from single cells were not different from control values representing pure cloned cDNA of one type. The results are consistent with the conclusion that there is complete exclusivity of expression of one L subtype in each of the L cones of this one male.

There were some cells that did not produce as clean a result. The observation of a minor second band can be explained either by inefficient cutting of the restriction enzyme or by contamination of amplified mRNA from other lysed cones that was collected along with the single cell. However, our results cannot rule out the possibility that a very small amount of a second pigment gene is being expressed in a subset of the cells.

#### 4. DISCUSSION

Techniques of single-cell isolation and single-cell RT-PCR were used to characterize the visual pigment gene expressed in individual cone photoreceptor cells and to count the relative numbers of cone types and subtypes in the human retina. Our studies were designed to answer questions about gene expression and to determine whether there can be more than three cone types in the human retina.

From four male eye donors, 351 individual cones were collected. Out of the 351 cells, the pigment gene expressed in each of 256 cones was amplified by RT-PCR and characterized by restriction analysis. Our success rate of obtaining amplified PCR products from single cells for analysis was 72%. Cases in which we could not obtain a PCR product may have been caused by any of the following: The mRNA was degraded, the mRNA was in-

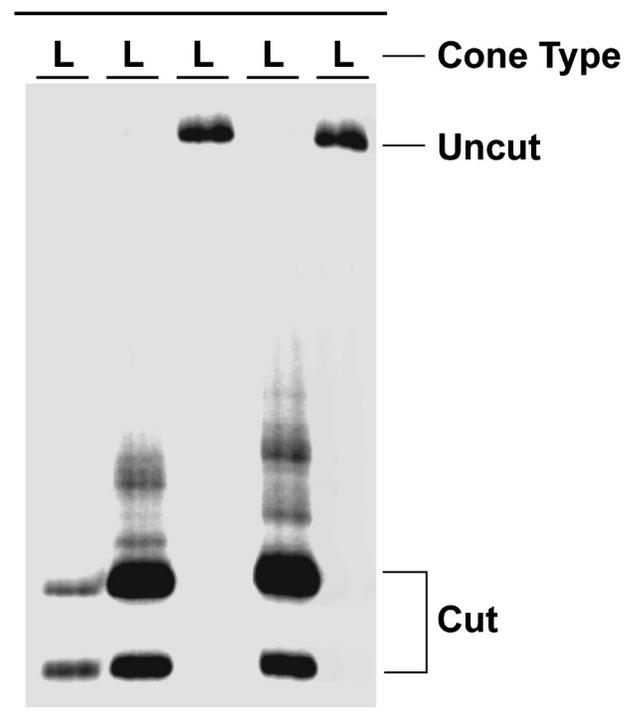


Fig. 8. PhosphorImage analysis of five single L cones from donor 54. Amplified L cDNA containing exon 3 was digested with BstYI targeting the nucleotide polymorphism at position 1026. Each lane represents a single cone cell. Two different subtypes of L genes are shown to be expressed. Three of the cone cells express the cut L gene fragment, and the other two cells express the uncut L gene fragment.

accessible to the reagents, the cone was lost or did not lyse, or the mRNA adhered to the side of the tube. In a few cases, there was a minimal amount of amplification, but the quality and quantity of the amplified DNA was so poor that we were unable to identify the product using the digestion assays.

We analyzed each of the 256 cone cells by RsaI restriction analysis to deduce whether the cone was L or M. Only one major X-linked pigment cDNA type was detected from each cell, from either an L gene or an M gene. The single-cell analysis allowed us to examine the exclu-

sivity of pigment gene expression. The limitations of this technique in answering questions of exclusivity include errors that could be introduced by incomplete enzyme digestion and the introduction of small amounts of contaminating mRNA from lysed cells carried along with a single cell when the cell is collected. Nonetheless, results from the examination of selected cells is consistent with the idea that there is expression of one pigment gene per cell and that the expression of that one gene completely excludes the expression of other photopigment genes. These experiments do not address the possibility that more than one gene can be expressed at early stages of development<sup>20</sup> or that occasionally an indecisive cone may express two pigments.<sup>21</sup> For a subset of the cells for which we could not get a PCR product using the L/M primers, we did get a product with the S cone pigment DNA-specific primers. This result suggests that the S cones that we identified did not express L or M pigment. However, the cones that did have L or M expression were not tested for the presence of S cDNA. Thus, we cannot rule out that some cones that express L or M also coexpress S.<sup>22</sup>

The L cones outnumbered the M cones in the four subjects that we examined. This is consistent with earlier mRNA studies that have demonstrated that L pigment gene expression usually predominates.<sup>14</sup> The results are also consistent with conclusions from psychophysical and imaging studies, which indicate that there are usually more L cones than M cones in the human retina.<sup>23–27</sup>

The X-linked pigment gene arrays are complicated, and there are widespread individual differences. On the basis of the finding of more than one species of L cone pigment mRNA in homogenates of retinal tissue, the cones of subject 54 were screened by restriction analysis for expression of multiple L genes. The majority of his L cones expressed one L pigment gene type that corresponded to the first gene in his array. However, a small population of cones exclusively expressed a second subtype of L cone pigment.

For two individuals, each cone that did not express an M or L gene was screened for S pigment gene expression (this experiment was not done for the other two subjects). The number of S cones in the retina was 8% for subject 54. In a smaller sample of cones, subject 46 had 13% S

cones. The percentages are not significantly different from each other ( $p = 0.31$ ). These results are consistent with those from immunohistochemistry.<sup>19</sup>

The single cones were taken from a midperipheral patch of retina. Studies have shown that the M:L mRNA ratio decreases with increasing eccentricity; however, the significant change occurs outside the midperiphery where these cones were collected.<sup>14,17</sup> The cones were taken from the same midperipheral location across all subjects to avoid problems in interpretation that might occur from differences in cone populations at different eccentricities. For each of the four eye donors, the M:L cone pigment mRNA ratio was estimated from the same retinal location as the collected single cones. There was good correspondence between the relative ratio of M:L pigment mRNA and the relative ratio of M:L cones in these retinas. These results suggest that M and L cones contain approximately equal amounts of message and thus the mRNA ratios can be used to estimate of the ratios of L:M cone populations in the retina.

Knowing the number of different cone types and their relative proportions is fundamental to understanding the biological basis of human vision. Results from experiments on retinal extracts together with single-cell studies make it clear that more than just two X-linked pigment genes can be expressed in some men. The gene positioned first in the array is usually expressed in the highest number of cones. The pigment genes downstream of the first gene are expressed in fewer cones. When more than two genes are expressed, each gene is represented by its own subpopulation of cones.

This work demonstrates that men can occasionally have the photoreceptor basis for tetrachromatic color vision. Normal human color vision is presumed to be uniformly trichromatic. If males are uniformly trichromatic, as usually presumed, then we are left with the conclusion that the addition of the second spectral type of L cone does not extend normal color vision to tetrachromacy. It is intriguing that the two L cone subtypes are well enough separated spectrally that if they were wired appropriately they could provide an ample additional color signal. This is confirmed in men with the color vision deficiency deuteranomaly. They are missing normal M cones, but they have red–green color vision that is

**Table 6. PhosphorImage Analysis of Single L Cone Cells Digested with Bst YI**

Cell Number	Uncut	Cut 1	Cut 2	Uncut/Cut	Proportion Uncut	Cone Type
1	$6.61 \times 10^2$	$1.59 \times 10^5$	$1.19 \times 10^3$	0.0042	0.0041	L1
2	$5.47 \times 10^2$	$7.69 \times 10^4$	$1.24 \times 10^3$	0.0070	0.0069	L1
3	$6.04 \times 10^1$	$6.04 \times 10^1$	$3.27 \times 10^4$	0.0018	0.0018	L1
4	$2.80 \times 10^2$	$6.20 \times 10^2$	$1.98 \times 10^5$	0.0014	0.0014	L1
5	$3.65 \times 10^2$	$1.53 \times 10^5$	$1.95 \times 10^4$	0.0021	0.0021	L1
Cut Control	$2.02 \times 10^3$	$1.12 \times 10^5$	$1.33 \times 10^5$	0.0082	0.0082	
6	$8.31 \times 10^4$	$1.27 \times 10^2$	$2.59 \times 10^2$	215.6	0.9954	L2
7	$1.72 \times 10^4$	$1.16 \times 10^2$	$1.41 \times 10^2$	66.71	0.9852	L2
8	$3.67 \times 10^4$	$1.55 \times 10^2$	$2.48 \times 10^2$	90.97	0.9891	L2
9	$2.49 \times 10^5$	$1.86 \times 10^2$	$2.37 \times 10^2$	589.1	0.9983	L2
10	$2.95 \times 10^5$	$1.94 \times 10^3$	$7.91 \times 10^2$	108.1	0.9908	L2
Uncut Control	$2.30 \times 10^4$	$5.92 \times 10^1$	$4.27 \times 10^1$	170.8	0.9955	

based on two subtypes of L cones. The deuteranomalous who have L subtypes with spectral separations that are similar to those that can occur in normals use them to derive remarkably good color vision.<sup>28</sup> The two L pigments that provide the third dimension of color vision in deuteranomalous trichromats could, in principle, endow an equally robust fourth dimension of color vision to an occasional man with normal color vision. Therefore it appears that human color vision is not limited to trichromacy by the presence of only three types of cones: It is possible that some humans may be tetrachromats. Tetrachromatic humans would number only a few men but could include a large proportion of women.<sup>18</sup> On the other hand, if humans are limited to trichromacy, the bottleneck where the outputs from multiple cones are reduced to three channels must occur at a higher level of the visual pathway.

## ACKNOWLEDGMENTS

This research was supported by National Institutes of Health grants EY09303, EY09620, and EY01921 and by Research to Prevent Blindness. We thank T. Trusk for his technical expertise in designing the single cone collection procedure and P. M. Summerfelt for her help in preparing the manuscript and the figures.

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