



Supporting Online Material for
**Emergence of Novel Color Vision in Mice Engineered to Express
a Human Cone Photopigment**

Gerald H. Jacobs,* Gary A. Williams, Hugh Cahill, Jeremy Nathans

*To whom correspondence should be addressed. E-mail: jacobs@psych.ucsb.edu

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Materials and Methods

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The design of the human L cone pigment knock-in mouse has been described (S1). In short, most of the coding sequences for the native mouse M cone pigment ($\lambda_{\max} = \sim 510$ nm) were replaced with sequences encoding a human L pigment containing a polymorphic alanine at position 180 (yielding a pigment with a λ_{\max} of ~ 556 nm). The knock-in construct encodes an M/L hybrid pigment in which the C-terminal 80%, including the regions responsible for the spectral difference between M and L pigments, are derived from the L pigment. Subsequently these mice were backcrossed for five generations into a C57BL/6 background.

Electroretinogram Measurements

Details of the procedures for recording the mouse ERG appear elsewhere (S2). ERGs were recorded from mice anesthetized with an IM injection of a mixture of xylazine hydrochloride (8 mg/kg) and ketamine hydrochloride (42 mg/kg) using bipolar ring electrodes that contacted the surface of the eye through a tear film. All recordings were done in a room illuminated to yield ~ 150 lux at the cornea of the test eye. Stimuli were derived from a three-beam optical system. The outputs from the three beams were imaged in register through a dilated pupil onto the retinal surface in Maxwellian view as a 59 deg circular spot.

Spectral sensitivity measurements were made using a flicker photometric procedure (S3) in which a flickering test light (12.5 Hz) derived from a monochromator (50 W tungsten-halide lamp, 15 nm half-bandwidth) was temporally interleaved with a similarly-flickering, achromatic reference light (3700 td) that originated from a tungsten-halide source. The fundamental components of the responses elicited by the two lights, each individually based on the averaged amplitude of the response to 50 stimulus cycles, were electronically compared and the intensity of the test light was iteratively adjusted by changing the position of a neutral-density wedge until the

responses were equal. The intensity of the test light at the point of equation yields one point on a spectral sensitivity function. Such equations were made for test lights spaced at 10 nm intervals across the spectrum. The total extent of the spectrum sampled varied dependent on the mouse pigment phenotype. Two complete spectral scans were made and the results obtained from these were subsequently averaged. Flicker photometric equations made following this procedure are typically repeatable to a variation of < 0.04 log units (S3). ERG spectral sensitivity functions were fitted using photopigment absorption functions (S4) that were progressively shifted along the wavelength axis (in steps of 0.1 nm) until the best least squares fit was obtained. In establishing these fits, pigment optical density was treated as a free parameter (with obtained values usually <0.1).

Voltage vs intensity (V-log I) functions were obtained from mice expressing either M or L cone pigment. To accomplish this, the fundamental component of the response to 12.5 Hz flicker was recorded. The stimulus was created by passing light through a 455 nm long-pass filter thus minimizing any contribution resulting from absorption of light by the mouse ultraviolet-sensitive (UV) photopigment. Responses were recorded for a series of intensities increasing in steps of 0.2 or 0.3 log units from response threshold to saturation. At each of the intensity values five separate response amplitudes were averaged, each of which in turn consisted of 50 presentations of the test light. The averaged V-log I functions were best fitted using the hyperbolic equation: $V(I) = V_{\max}[I^n/(I^n + K^n)]$, where K is the intensity required for half-maximal response, n is the slope, and V_{\max} is the saturation voltage (S5).

Cone Photoreceptor Labeling

Cone labeling was performed on four adult mice, two expressing the M pigment and two expressing the L pigment. Animals were euthanized with an overdose of halothane. Each eye was marked for orientation, enucleated, and the cornea and lens were removed. The eyecups were immersion fixed in 4% paraformaldehyde diluted in a NaPO₄ buffer solution (0.086 M, pH 7.3) and whole retinas were dissected from the eyecups.

The labeling procedure is described elsewhere (S6). In brief, retinas were incubated in a combination of biotinylated peanut agglutinin (PNA) to label the cone matrix sheaths and JH492, a rabbit antiserum that selectively labels photoreceptors containing M or L opsin (S7). The retinas were incubated with secondary probes (streptavidin/goat anti-rabbit IgG) conjugated with fluorescent labels (Texas Red and fluorescein) and subsequently flat mounted photoreceptor side up on slides. Digital images (0.029mm^2) were collected at 1 mm intervals across the retina using a fluorescence microscope equipped with a CCD camera. The images were adjusted in brightness and contrast using Adobe photoshop and labeled cells were counted to obtain estimates of the average cone densities for each retina.

Behavioral Tests

A. General Techniques

The visual capabilities of mice were established in a three-alternative, forced-choice discrimination task. The test apparatus and the general procedures used for this purpose were described earlier (S8). From inside a small test chamber the mice viewed stimuli presented on three circular panels mounted in a horizontal line along one wall of the chamber (2.5 cm diameter with center-to-center separations of 5 cm) that were transilluminated by lights drawn from an optical system located outside the chamber. The test light (the positive stimulus) was derived from a grating monochromator (half-energy passband = 16 nm) equipped with a 75 W xenon lamp. An automated mirror system directed this light to any of the three panels. Light from a second source, a 150 W tungsten-halide lamp, was used to diffusely and equally illuminate all three panels. Depending on the goals of the experiment, on any given trial the test light was either added to the background light (increment-threshold measurements) or replaced it (tests of color vision). During all of these experiments the interior of the test chamber was diffusely illuminated (mean illuminance = 100 lux).

Through an operant shaping procedure mice were trained to signify on which of the three panels the test light appeared by touching that panel. Correct choices were reinforced by delivery

of 0.028 ml of a highly palatable fluid (Soymilk) through feeding tubes located just above each of the panels. Over trials the location of the test light was varied randomly across the three panel positions. The nature of the difference between the light illuminating the positive panel and the other two panels was systematically varied to permit assessments of several visual capacities, the details of which are noted below. Test trials were signaled by a concurrent cueing tone and terminated at the time of response or after 15 sec without a response. The intertrial interval varied somewhat dependent on the response proclivities of each subject, but averaged about 6 seconds. A noncorrection procedure was followed. All aspects of stimulus presentation, reinforcement delivery, and response monitoring were computer controlled. The mice were food deprived for ~21 hours prior to the test session and then fed following the session in an amount sufficient to maintain weight at a normal level. Animals were tested daily, typically completing between 300 and 500 trials/test session.

B. Increment-Threshold Measurements

Increment thresholds were measured in eleven mice for two test wavelengths (500 nm and 600 nm). For this purpose the three stimulus panels were steadily illuminated with an achromatic light (5350 K; luminance = 20 cd/m²), and on each trial the monochromatic test light was added to one of the panels. Across trials the intensity of these lights was varied in steps of 0.3 log units descending from a level where the animals showed high levels of discrimination (typically, 80% correct or better) down to an intensity that yielded chance performance. Discrimination results were accumulated until at least 100 trials had been run at each of the wavelength/intensity combinations. Psychometric functions were constructed from these results using a logistic function having asymptotes of 100% and 33% correct with the variance and mean as free parameters. The function providing the best fit to this data array was determined and thresholds were computed as the stimulus intensity required to yield performance corresponding to the 95% upper confidence level.

C. Brightness Controls in the Color Tests

To render brightness irrelevant as a cue for discrimination in this test we followed a procedure devised earlier for this purpose (S2). Each mouse was first extensively trained in the increment-threshold task described above where they learned to reliably (asymptotic levels of 80% correct or greater) pick the brighter of two lights. Once this was accomplished, the test light was set to 500 nm; the other two panels were illuminated with 600 nm light (half-energy passband = 10 nm; panel luminance = 79.2 cd/m²). On each test trial the test light now replaced the background light rather than being added to it as in the previous experiment. To obtain a brightness equation between the two wavelengths, the intensity of the test light was progressively diminished in steps of 0.2 log units. When the test light was much brighter the animal selected it consistently, but as the test light intensity was decreased the percentage of correct selections declined and then, eventually, dropped below chance, i. e., the animal began to select the standard light, presumably because it now appeared as the brighter of the two. The intensity value of the test light at the point where discrimination performance passed through the chance level (33%) was taken as defining equal brightness for the two lights. Similar equations were obtained for other combinations of test and standard wavelengths. Brightness equations for wavelengths that were intermediate to those for which direct matches were made were approximated by reference to the ERG spectral sensitivity functions previously obtained from each animal. As demonstrated earlier, this procedure can be used to rather tightly define brightness matches in mice (S2).

D. Wavelength Discrimination

Once brightness equations were obtained, we attempted to then train animals to discriminate between equally bright monochromatic lights. To do this, in each test session the two monochromatic lights (test and standard) were presented at the point of calculated equal brightness and, on randomly interleaved trials, with the test light set so as to be either more or less bright than the standard light. These intensity variations were stepped in 0.1 log unit increments above and below the equal brightness value so as to cover a total range of ± 0.5 log units. In successive experiments wavelength discrimination was measured against a total of seven standard

lights: 570, 580, 590, 600, 610, 620, and 630 nm. The luminance of these standards varied slightly, covering the range from 64-79 cd/m². In each case, the test light was first set to 500 nm or 510 nm and, if that discrimination was successful, its value was progressively stepped toward the wavelength of the standard in steps of 5 or 10 nm.

E. Color Matching

To derive color matches, light from a color mixer was used as the source illuminating the positive stimulus panel. This color mixing device was constructed by mounting two monochromatic filters (half-energy passband = 10 nm) side-by-side on a linear positioner. Light from a 150 W tungsten halogen source was beamed through these filters. A programmable stepper motor was used to shift the positioner, thus progressively changing the proportion of the light beam that passed through each of the two filters. The overall intensity of mixture was varied with a programmable neutral-density wedge. In this test the other two stimulus panels were illuminated with monochromatic light generated as described above. All other details of the experiment were the same as those employed in the wavelength discrimination tests, including the methods used to control brightness cues. The luminances of the two standard lights (580 and 600 nm) were 63 cd/m² and 79 cd/m² respectively.

Supplemental References

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