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Microscopy of multiple visual receptor types in *Drosophila*

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Purpose: To take advantage of specialized microscopic methods and transgenic stocks, to understand the properties of each rhodopsin now that *Drosophila*'s six rhodopsins (Rh1-Rh6) have been isolated.
Methods: The visual pigment containing organelles, the rhabdomeres, were imaged in live flies with the pseudopupil in standard and confocal microscopes. Five transgenic *Drosophila* strains in which Rh2-Rh6 replaced the native Rh1 in R1-6 receptors were compared with normal controls (Rh1 in R1-6) for two lines of work: (1) autofluorescence of rhodopsin; and (2) imaging rhodopsin. Other transgenic *Drosophila* in which the Rh1, Rh3, and Rh4 promoters drive the green fluorescent protein (GFP) reporter were used for other purposes, especially distinguishing the R7/8 types.
Results: We show, for the first time, that visual pigment appears pink in white light, especially for Rh1 and Rh6. While showing that rhodopsin-metarhodopsin conversions were understood by their respective wavelengths, we discovered that, for Rh6, rhodopsin and metarhodopsin could not be spectrally separated. Relative fluorescent emission, Rh1=Rh5>Rh6>Rh2>Rh4>Rh3, was of little value in explaining differences between bright and dim autofluorescence in R7. Rather, analysis of GFP driven by Rh3 and Rh4 promoters show that the rhabdomeres with bright autofluorescence are the ones that contain Rh4.
Conclusions: Careful imaging provides a useful approach to analyzing *Drosophila* rhodopsins. Amid a considerable body of microscopic data, we identify the sources of bright and dim R7 rhabdomeres, and we demonstrate the unique properties of Rh6.

The fly, with its two compound eyes and three simple eyes (ocelli), has remained a model to investigate multiple spectral receptor types for decades. Each ommatidium in the compound eye has 8 photoreceptor cells of 3 anatomical types. On the basis of genetic dissection in *Drosophila*, R1-6 (R1-R6) were shown to be sensitive to blue and ultraviolet (UV) light, while R7 is a UV receptor and R8 is a blue-green receptor [1]. Multiplicity beyond this level for R7 and R8 was first shown by fluorescence microscopy and electrophysiological data in the housefly *Musca*: Some R7 rhabdomeres fluoresce yellow (R7y), while others are pale (R7p) [2]. Two R8 types were also found.

Drosophila has contributed uniquely to this research. R1-6 express one blue sensitive opsin, Rh1 [3,4]. The gene for a second opsin, Rh2, was isolated [5] and shown to be expressed in ocelli [6,7]. Two R7 opsin genes have been isolated in *Drosophila melanogaster*, Rh3 [8] and Rh4 [9]. These genes are expressed in non-overlapping subsets of R7 cells [10]. In *Drosophila*, both are UV rhodopsins [11]. Homologous opsins were found in related species including the blowfly *Calliphora* [12], *Drosophila virilis* [13], and *Drosophila simulans*, *pseudoobscura*, *virilis*, and *mercatorum* [14]. When the gene for Rh5 was isloated [15,16], it was found to code for rhodopsin in those R8 rhabdomeres that are proximal to R7 rhabdomeres that contain Rh3; likewise, the gene for Rh6 [17] codes for rhodopsin in R8 rhabdomeres that are proximal to Rh4 containing R7 rhabdomeres [18].

The confirmation in *Drosophila* of bright and dim R7 autofluorescence emissions, made possible by confocal microscopy [19], led us to this more thorough investigation using various microscopic techniques. R1-6 predominates in many assays of the visual system. Rh2-Rh6, by contrast, are relatively minor and more difficult to analyze. For this reason, we took advantage of the availability of transgenic flies with each minor opsin (Rh2-Rh6) replacing the predominant opsin (Rh1) in R1-6. Also, we obtained flies in which the promoters of Rh1, Rh3 and Rh4 drive green fluorescent protein (GFP) reporter into their respective retinula cells. Utilizing these strains, we could compare R1-6 in the pseudopupil, an image of the photoreceptors [1], to examine rhodopsin specific fluorescence and rhodopsin conversions. Experiments with *Drosophila* reared on different diets demonstrate the facility of pseudopupil analysis in live fly eyes; this should expedite promoter-reporter analyses of the transcriptional control of opsin by retinoids. Microscopic techniques applied to the eyes of live flies are useful, and the results are presented in the context of an overall functional anatomy.

METHODS

Animals: White eyed otherwise wild type flies (*w*, the positive control) had been maintained in this laboratory for decades. Two negative controls are deficient in R1-6 opsin: *w*;*ninaE*^{ora} [20] (maintained in this laboratory since the work of Harris et al. [1]) *w*;*ninaE*^{ol17} [3] (obtained from Justin Kumar then in Prof. Donald Ready's laboratory at Purdue University, West Lafayette, IN).

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For convenience we refer to the white eyed transgenic stocks with short nicknames, *w;Rh(1+2)*, *w;Rh(1+3)*, *w;Rh(1+4)*, *w;Rh(1+5)*, and *w;Rh(1+6)*. (1) Feiler et al. [21] generated transgenic *Drosophila* expressing Rh2 in R1-6; (2) Feiler et al. [11] made flies with Rh3 and Rh4 in R1-6; (3) Chou et al. [15] made flies with Rh5 in R1-6; and (4) Britt [22] made flies with Rh6 in R1-6. White eyed constructs were on a background of $ninaE^{oI17}$, a deletion of the gene for the native R1-6 opsin, Rh1 [3]. The Rh1 promoter was fused to each of the Rh2, Rh3, Rh4, Rh5, and Rh6 coding sequences to drive the ectopic expression into R1-6. Three transgenic fly stocks were obtained from Dr. Robert Hardy in the laboratory of Prof. Charles S. Zuker, University of California-San Diego: (1) w^{1118};*Rh(1+2)#24;sr* $ninaE^{oI17}$ e^{s}; (2) *w;Rh(1+3)#1;*$ry^{506}$$ninaE^{oI17}$; and (3) w^{1118};*Rh(1+4)#1;*$ninaE^{oI17}$ e^{s}. The *ninaE* opsin gene is on third chromosome; The P-elements with the Rh1 promoter fused to the Rh2, Rh3, and Rh4 coding sequences are on the second chromosome. From Prof. Steven Britt, University of Colorado HSC Denver, we obtained three stocks (called 153D, 14C, and 64A) of *y w;;sr* $ninaE^{oI17}$ with an Rh(1+5) P-element marked with y^{+} in three different locations and five such stocks (17A, 78A, 87A, 107A, 151A, and 159A) for Rh(1+6) [15]. The different *w;Rh(1+5)* and *w;Rh(1+6)* strains gave very similar results. White (*w*) is on the first (X) chromosome; *e*, *ry*, *y*, and *sr* are markers.

Stocks with the green fluorescent protein (GFP) reporter driven by rhodopsin promoters were obtained from Dr. Franck Pichaud (now at University College London, London, United Kingdom) and Prof. Claude Desplan (New York University, New York, NY). For Rh1, the UAS (upstream activating sequence) Gal4 system was used. The F1 from a cross between w^{-}/w^{-}; *Sp/CyO*; *Rh1Gal4 p(17)/Rh1Gal4 p(17)* (Rh1 promoter driving Gal4 in a rosy plasmid homozygous on the third chromosome) and w^{-}/w^{-}; *UAS GFP/UAS GFP*; *UAS GFP/UAS GFP* (homozygous for UAS GFP on second and third chromosomes) worked well. For Rh3, we used w^{-}/w^{-}; *Rh3GFP/CyO;;MRRS;TM2* (here a minimal Rh3 promoter, starting at -243, drove Rh3 fused to GFP [23]) and for Rh4, we used two stocks w^{-}/w^{-};*Sp/CyO*;*Rh4GFPp*(w^{+})*/TM2* (where a minimal Rh4 promoter, -373 to +85, drove GFP, and with different locations of Rh4GFP on the third chromosome for the two stocks [23]) where the "*Rh3GFP*" and "*Rh4GFP*" terminology is meant to designate the Rh3 promoter or the Rh4 promoter driving GFP.

In addition to examining *Drosophila melanogaster*, we made comparisons with another species (*Drosophila virilis*), and another genus (*Musca domestica*). White eyed *Musca* were obtained from Michael Zimmerman in Prof. Joseph O'Tousa's laboratory at the University of Notre Dame, South Bend, IN. We obtained white and red eyed *Drosophila virilis* from the Mid-America Drosophila Stock Center (Bowling Green, OH). Curiously, the white eyed stock lacked R7 [24], so a cross was performed to construct a white eyed stock that did have R7 using the deep pseudopupil to screen for R7.

Drosophila were raised on a standard medium with enough carotenoids for fully developed visual function; for comparison, *Drosophila* were reared on Sang's medium for thorough retinoid deprivation [19,25]. For some studies, a yeast-glucose food was used because, though it lacks chromophore precursors, it does activate the rhodopsin gene [25]. Flies were reared in a 12 h light/12 h dark cycle of fluorescent lighting at room temperature. *Musca* larvae were reared on a recipe of 340 g fly larvae medium (formerly CMSA medium; PMI Feeds, St. Louis, MO), 5 g active (live) dry yeast, and 10 cc of 50% sucrose in 750 ml of liquid; to ensure carotenoid sufficiency, the liquid was 50% carrot juice. Adults were maintained on water, milk, carrot juice, and sugar.

Pseudopupil preparation: Heads of living flies were attached to the glass microscope slide to expose the dorsal region with clear nail polish (L'Oreal Top Coat, Cosmair, Inc., New York, NY); their bodies were stabilized using dental wax (ESPE Protemp Bis-Acryl composite resin, Norristown, PA; Figure 1). Because of the regularity of receptor structure in each ommatidium and of the angle between ommatidia, there is a magnified virtual image about 80 μm in diameter of the rhabdomere tips superimposed from all the ommatidia sampled (typically 25, depending on the numerical aperture of the microscope objective) about 150 μm behind the surface of the eye; this is the deep pseudopupil (Figure 2). Deep pseudopupils in *Drosophila* were viewed with a 10x 0.25 NA or a 16x 0.25 NA achromat objective by illuminating the back of the head with a narrow beam of light [26].

Visualizing rhodopsin and rhodopsin-metarhodopsin conversions: For rhodopsin-metarhodopsin conversions, actinic light was applied through the fluorescence epi-illuminator of a Leitz Dialux microscope (now Leica, Inc., Deerfield, IL). The fluorescence epi-illuminator and a 100 W Mercury arc were used with either the A cube, to provide UV excitation and full visible spectrum viewing, or the H cube, for blue excitation, and green and red viewing. Corion (Holliston, MA) or Edmunds (Barrington, NJ) interference filters in the transmitted beam and in the incident illuminator were used. Neutral density filters in the excitation beam controlled intensity so that maximum conversion occurred in 1 to 10 s. The rhodopsin-metarhodopsin conversion was witnessed as a darkening of the deep pseudopupil [26] viewed with transmitted light when the actinic beam was turned on. If the actinic beam was too bright, fluorescent emission added to the intensity; fortunately, when the actinic beam was dimmed sufficiently with neutral density filters, the darkening was not diminished. An Optronics (TEC-470, Goleta, CA) camera was very useful because the integration time could be set to see images that were too dim for the naked eye. We needed special refinements to see the color of rhodopsin directly. Even when the camera was carefully white balanced, and the A cube was in use, fly eyes looked a dingy yellow, so a broad band blue filter was selected to whiten the beam. The camera fed into a Sony Color Video Printer (UP-5200MD) that fed, in turn, into a Sony monitor (Trinitron model PVM-1343MD). Images were digitized with a Coreco Oculus TCX/MX frame grabber (St. Laurent, Quebec, Canada) in a personal computer using Image Pro Plus 3.0 software (Media Cybernetics, Silver Spring, MD).

Confocal microscopy: N. Franceschini showed one of us

(WSS) his technique to visualize fluorescence of individual *Musca* rhabdomeres with a standard fluorescence microscope in 1978 (placing a small iris in the trinocular head of the microscope in the optical path between the fly head and the camera). That method did not work on *Drosophila* despite numerous attempts. That was the reason we first applied confocal microscopy to the pseudopupil of *Drosophila*. The laser scanning confocal microscope at the Laboratory for Optical and Computational Instrumentation (LOCI) was used for this and earlier [19] research. A Bio-Rad (Hercules, CA) MRC-600 confocal microscope was fitted with a Kr/Ar laser on a Nikon Optiphot. The deep pseudopupil was viewed with a 10x Plan apo dry objective, NA=0.45. Rhabdomere tips were viewed directly using "optical neutralization of the cornea" with immersion oil and an oil immersion 60x Plan apo objective NA=1.4. Excitation at 488 nm was provided by optics intended for fluorescein. Earlier [19] it was shown that, in the MRC-600 confocal microscope, R1-6 plus R7 were visible with fluorescein optics, while only R1-6 fluoresced with rhodamine optics. Since we were interested in R7 properties, we used only fluorescein optics in this study. Image acquisition was operated using Bio-Rad's COMOS software. Images were also analyzed using NIH Image, version 1.61, running on Macintosh OS 8.6 and above. One of our most useful techniques, and a special capability of the confocal microscope, was to collect about 10-30 optical sections through a z focus series of up to 100 μm and convert this into an animation. During the course of this research, the computer analysis developed considerably. A program developed by CFT at LOCI, 4D Turnaround (version 3.24) for the Macintosh, converted stacks to Quicktime movies that were analyzed with NIH Image. For the PC, Confocal Assistant assisted in image analysis.

Calibration: Fluorescence microscopy utilizes extremely intense beams. For white eyed *Drosophila*, fluorescence would typically be observed upon excitation with about 10^{18} quanta/cm^2/s of blue light [27]. Intense short wavelength light not only excites a vitamin A dependent fluorescence in the deep pseudopupil of white eyed *Drosophila*, but also induces the formation of a fluorescent product of the vitamin A based visual pigments [28]. If the amount of blue light (product of intensity and time) is sufficient, about 10^{20} quanta/cm^2, there is a 50% decrease in visual pigment and a 50% increase in this fluorescent product [29], and an eventual photoreceptor degeneration [30]. By visual inspection, the beam of the confocal microscope seemed about as intense as that of the standard fluorescence microscope used in earlier work. It was hypothesized that this impression was based on a much more intense but much smaller spot of light rapidly rastering in the confocal microscope. With a model 268R head feeding into a model S370 optometer (United Detector Technology, Hawthorne, CA), the intensity of the excitation beam of the confocal microscope was determined. The fluorescein excitation cube uses a 488 nm filter. For the 10x objective, the intensity is approximately $5.95x10^{23}$ quanta/cm^2/s. This value takes into account a calculation of the diameter of the actinic beam [31]. In summary, the intensity of the small beam in the confocal microscope was several orders of magnitude greater than the intensity needed to excite fluorescence and create fluorescent products in earlier work on the deep pseudopupil of white eyed *Drosophila* utilizing the standard fluorescence microscope.

RESULTS

Background and controls: Optical sectioning, the confocal

Figure 1. *Drosophila* prepared for pseudopupil. White eyed *Drosophila* fixed to a microscope slide. This photograph was made with the help of Mr. H. Leertouwer in 1978 when WSS was on a research fellowship at Rijksuniversiteit Groningen, the Netherlands. The fly on the left was reared on the vitamin A replete diet, while the one on the right was vitamin A deprived. Note a slight off white coloring of the eye of the vitamin A replete fly, presumably due to visual pigments and possibly related with the pink seen in Figure 9. Flies need not be fixed to a slide; this is covered in the "Rhodopsin-metarhodopsin conversions" of the Discussion.

microscope's hallmark, optimizes obtaining a focus series (z-series). Franceschini and Kirschfeld [32], in introducing the *Drosophila* pseudopupil, photographed several focus planes from cornea to deep pseudopupil. Figure 3 extends this approach with an animation. At the start, the eye surface is bright in an area the diameter of the deep pseudopupil, seen at the end of the series. That distal brightness looks fuzzy with the 10x air objective. However, if it were viewed at higher power with immersion oil, that image would resolve into the individual rhabdomere tips seen with optical neutralization of the cornea.

Visual pigments in R1-6 plus R7/8 show vitamin A dependent fluorescence. R1-6 and R7 are bright in vitamin A replete *Drosophila* but dark in deprived flies (Figure 4). Only R7/8 fluoresce in Rh1 mutants *w;ninaE*^{ol17} and *w;ninaE*^{ora} (Figure 5). This is an important control because Rh1 is blocked in the transgenics using *ninaE* so that only the ectopic opsins (Rh2-Rh6) are expressed in R1-6.

Comparing bright and dim R7 tips observed in the confocal microscope: Figure 6 is confocal microscopy showing bright and dim R7s in the house fly (*Musca*) and the fruit fly (*Drosophila*), replicating and extending the findings of Franceschini et al. [2], using standard fluorescence microscopy. Because of the low radius curvature of the *Drosophila* eye, and the notable "optical sectioning" (low depth of focus) of the confocal microscope, only a narrow band of ommatidia

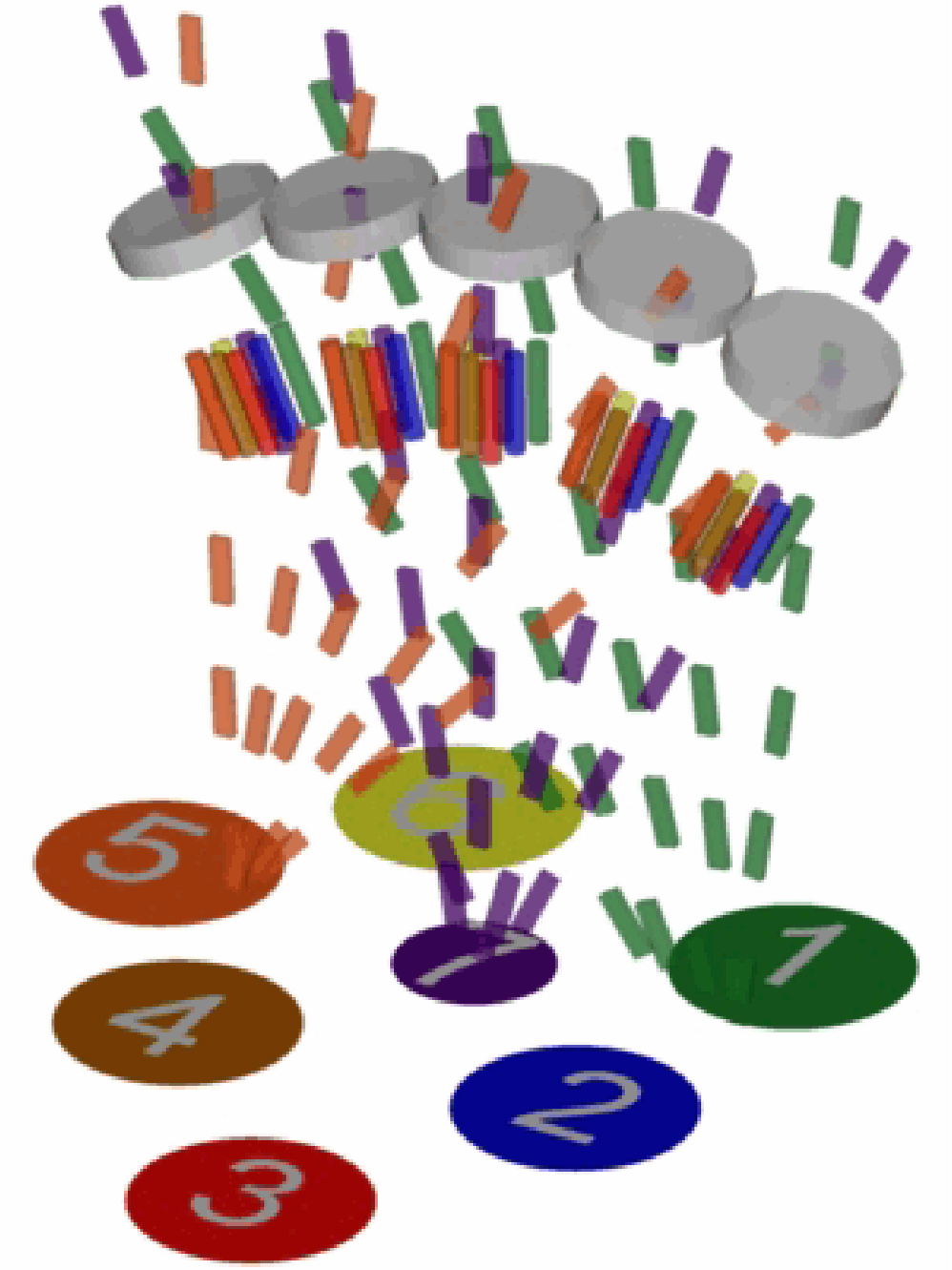

Figure 2. Diagram of deep pseudopupil. This diagram is redrawn from one used in the laboratory of Dr. Doekele G. Stavenga where WSS was a research fellow in 1978. The original was drawn by Jan Witpaard, a former student of Dr. Stavenga. This drawing helps to demonstrate the optics of the deep pseudopupil as characterized by Franceschini and Kirschfeld [32,47].

is in focus. Thus, we were concerned that R7's that were apparently dim might be slightly out of focus. For this reason, we obtained z series; focusing through the optimum plane, we repeatedly verified that dim R7s are not an artifact of poor focus.

Quantifying bright and dim R7's is hampered since few ommatidia are in one focal plane. Stepping through a z-series a few microns per step solved this limitation. Pooling from many z-series (from *w*, *w*;*Rh(1+2)*, *w*;*Rh(1+3)*, *w*;*Rh(1+4)*, *w*;*Rh(1+5)*, *Drosophila virilis* (white eyed), *ninaE*^{oI17}, and *ninaE*^{ora}), a 2.42/1 ratio was determined (of 3037, 1765 were bright, 730 were dim, and 542 could not be scored).

Autofluorescence of opsins Rh1 to Rh6 in normal and transgenic Drosophila*:*

Figure 7 compares the fluorescent deep pseudopupils of *w* controls with the five transgenics. The order of fluorescent emissions is Rh1=Rh5>Rh6>Rh2>Rh4>Rh3. Although we present typical micrographs, we have repeated these observations many times.

Ocelli: Since R1-6 in *w*;*Rh(1+2)* transgenics fluoresce, we sought fluorescence from ocelli directly. Because ocelli have no deep pseudopupil, we neutralized the ocellar lens and found spots of fluorescence in a narrow band of focus. A z-series revealed the fluorescence across the entire ocellus. Figure 8 shows an average through a z series. These punctate emissions were probably ocellar rhabdomeres based on the known size, number and distribution of ocellar rhabdomeres [33].

Visualizing rhodopsin-metarhodopsin conversions: Rh1 (R480) does not bleach, and its photoconversion with its stable metarhodopsin (M570) is easily seen in the deep pseudopupil [26]. Except for Rh6 (see below), we can visualize the rhodopsin-metarhodopsin conversions for all the rhodopsins. In Figure 9, Rh1 shows bright R1-6 with 579 nm light transmitted through the deep pseudopupil but dim R1-6 when 460 nm light

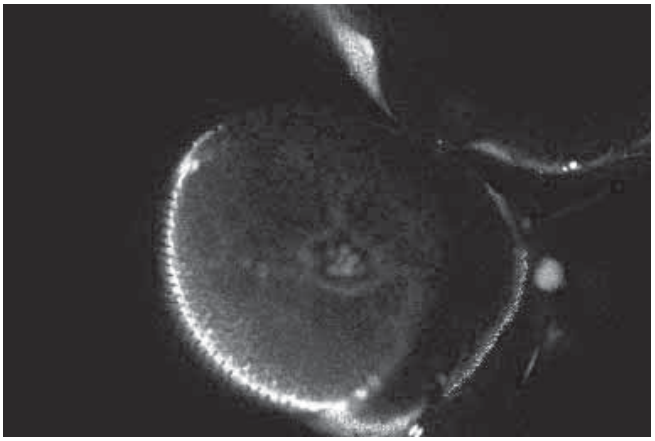

Figure 3. Focus from rhabdomere tips to deep pseudopupil. A representative frame from a confocal animation of a z-series of a "normal" Drosophila (white eyed, with Rh1 in R1-6) reared on vitamin A replete food. Note that there is a quicktime movie of this figure in the online version at the following URL: http://www.molvis.org/molvis/v10/a113/stark-fig3.html.

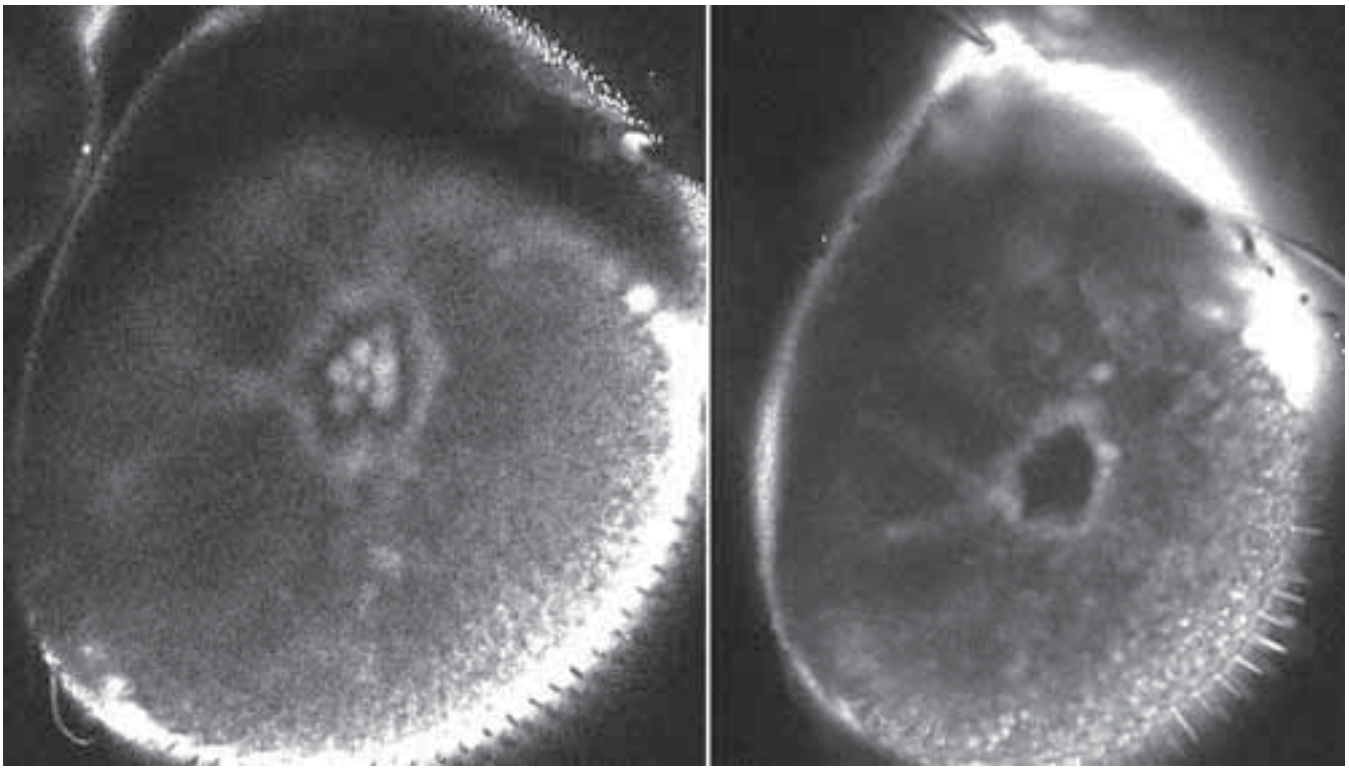

Figure 4. Deep pseudopupil and vitamin A. Confocal micrographs of the deep pseudopupil of white eyed normal (left) and vitamin A deprived (right) *Drosophila*.

converts 480 nm rhodopsin to 570 nm metarhodopsin. *w;Rh(1+2)* flies show bright R1-6 with 520 nm light transmitted through the deep pseudopupil but dim R1-6 when 436 nm light converts 420 nm rhodopsin to 520 nm metarhodopsin. *w;Rh(1+3)* flies show bright R1-6 with 480 nm light transmitted through the deep pseudopupil but dim R1-6 when 350 nm light converts 345 nm rhodopsin to 460-465 nm metarhodopsin. *w;Rh(1+4)* flies show bright R1-6 with 480 nm light transmitted through the deep pseudopupil but dim R1-6 when 376 nm light converts 375 nm rhodopsin to 460-465 nm metarhodopsin. *w;Rh(1+5)* flies show bright R1-6 with 505 nm transmitted through the deep pseudopupil but dim R1-6 when 405 nm light converts 437 nm rhodopsin to 494 nm metarhodopsin.

The situation for Rh6 is strikingly different from that of Rh1-Rh5. Interestingly, R1-6 remained continuously dark when transilluminated with 514 nm light (no additional adaptation, Figure 9, bottom right). Rh6 is the only one of six visual pigments with a shorter wavelength metarhodopsin (468 nm) than its rhodopsin (508 nm) according to Salcedo et al. [22]. The optical stimulator for viewing and converting visual pigment took advantage of fluorescence microscope optics, hiding the short wavelength actinic beam while viewing with a longer wavelength. Thus, *w;Rh(1+6)* were the only flies we viewed at a wavelength near rhodopsin's maximum.

Visualizing rhodopsin: Transilluminated with white light, Rh1 looks pink, while R7 looks relatively white. This pink appearance was also striking for Rh6; it was less for Rh5, then Rh2, and undetectable for Rh3 and Rh4 flies. The preferential transmission of long wavelengths is explained by a selective

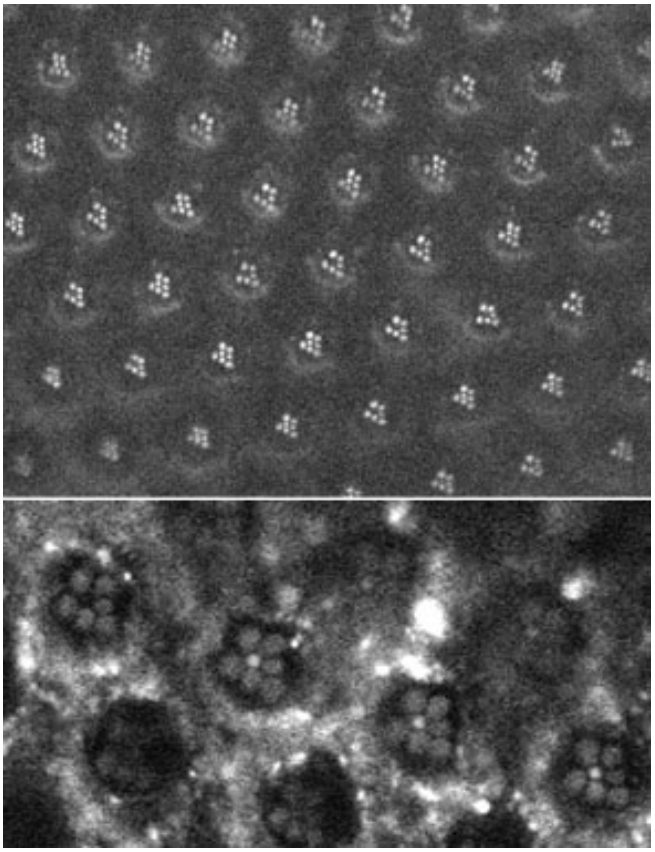

Figure 6. Rhabdomere tips. Confocal microscopy of optically neutralized cornea of white eyed *Musca domestica* (top) and *Drosophila melanogaster* (bottom). Note the bright versus dim R7s.

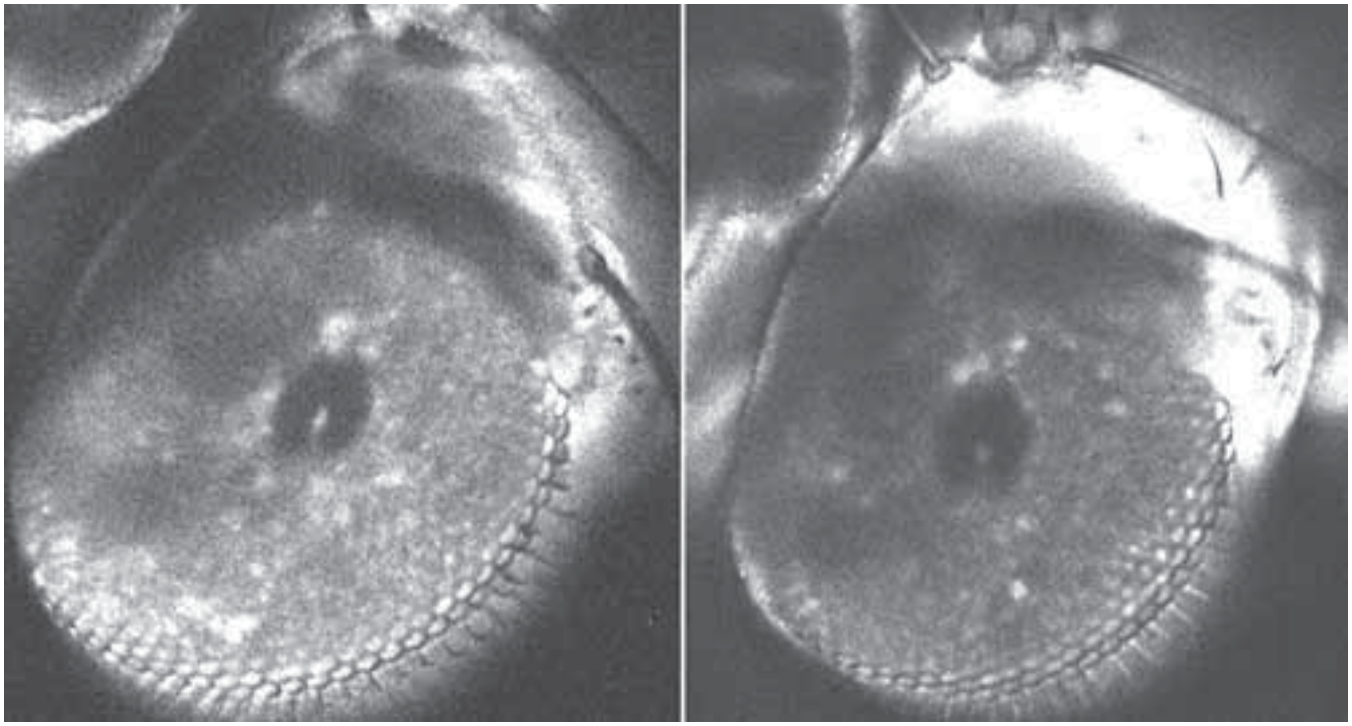

Figure 5. Mutants lacking rhodopsin in R1-6. Confocal image of deep pseudopupil of white eyed *ninaE*^{ol17} (left) and *ninaE*^{ora} (right). R7 but not R1-6 show fluorescent emission in these mutants that lack Rh1 in R1-6 (the negative control for transgenics with ectopic rhodopsins expressed in R1-6).

absorption of short and middle visible wavelengths which would be expected to be highest for Rh1 and Rh6.

Green fluorescent protein: Figure 10 shows green emission from GFP in R1-6 to 480 nm excitation (top left). White eyed flies allowed imaging of rhodopsin-metarhodopsin conversions. Note that the fluorescent image (left) is "fuzzy" (big or blurred) relative to the rhabdomere imaged with transmitted light (center). This implies that GFP is in cell bodies and in rhabdomeres. This is expected since GFP is not a membrane protein and since there is no mechanism to specifically target GFP to the membrane.

Figure 10 (second row) shows the same experiment on vitamin A deprived *Drosophila*. There is little or no GFP fluorescence, and, as expected, no darkening from rhodopsin-metarhodopsin conversion. GFP emission is variable in the negative controls and in the positive controls (top row); how-

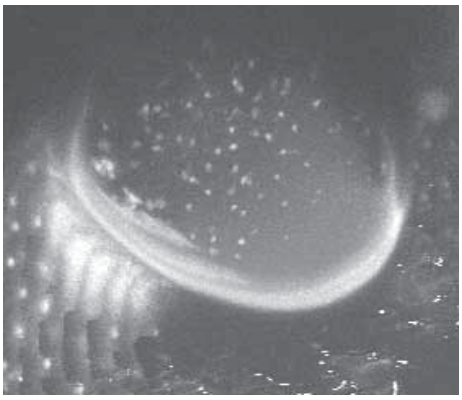

Figure 8. *Drosophila* ocellus in the confocal microscope. Optically neutralized cornea of an ocellus (simple eye) of white eyed otherwise wild type *Drosophila virilis*. The image was averaged from multiple "sections" in a z-series.

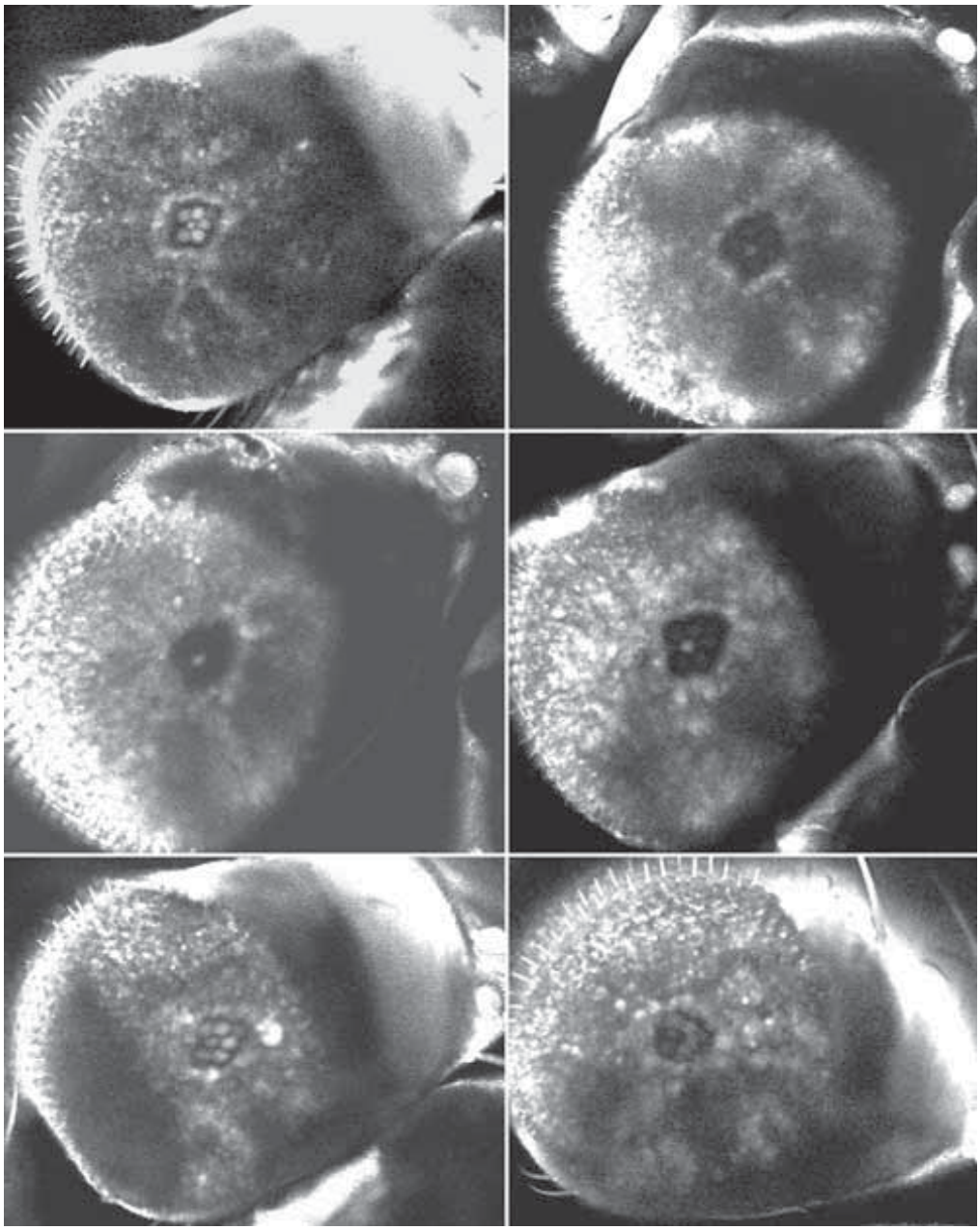

Figure 7. Confocal images of autofluorescence of normal and transgenic *Drosophila.* Fluorescent deep pseudopupil of vitamin A replete white eyed *Drosophila melanogaster* (fluorescein optics, BioRad 600). White eyed otherwise wild type (*w*, top left). These flies, in which the native Rh1 resides in R1-6, are compared with flies where the Rh1 promoter drives Rh2 to Rh6 into R1-6 (for ectopic expression). *w Rh(1+2)* (top right). *w Rh(1+3)* (middle left). *w Rh(1+4)* (middle right). *w Rh(1+5)* (bottom left). *w Rh(1+6)* (bottom right).

ever, the fluorescence is strikingly decreased in deprived flies, consistent with evidence that retinoids activate gene expression at the level of the opsin promoter [25,34]. Figure 10 (bottom row) shows this same experiment for a typical fly reared on a yeast food. This food has nutrients that were found to activate opsin gene expression but no precursors for chromophore necessary for rhodopsin function [25]. The fluorescence (left) and lack of darkening (compare middle and right)

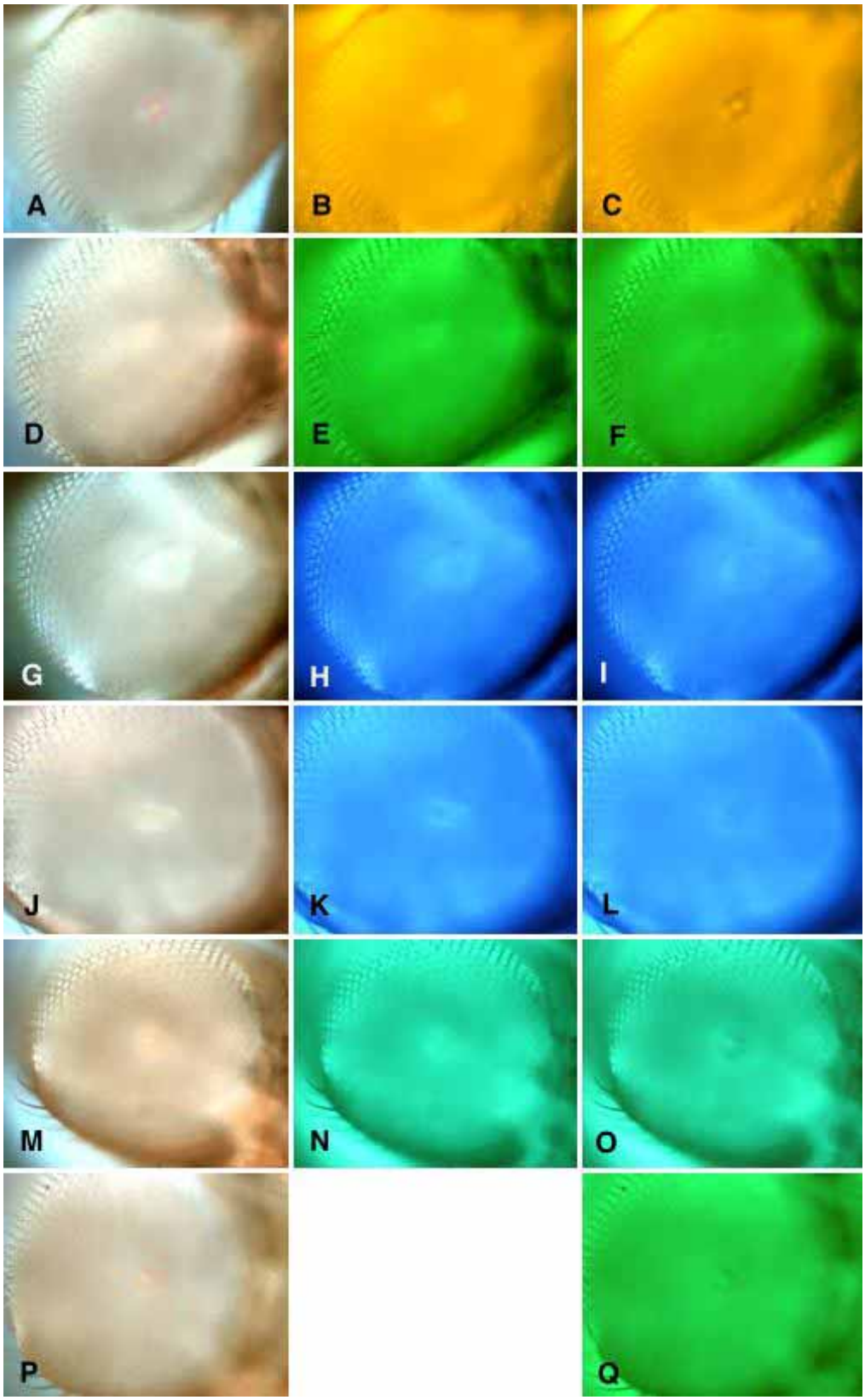

Figure 9. Imaging of visual pigment and rhodopsin-metarhodopsin conversions. The deep pseudopupil was used to image visual pigment and rhodopsin-metarhodopsin conversions in normal flies (*w*, row 1) and transgenic flies with Rh2 to Rh6 replacing Rh1 in R1-6 (rows 2 to 6), respectively. Native Rh1 in R1-6 viewed with white light (**A**), 579 nm light (**B**), and 579 nm light darkened with 460 nm light (**C**). Transgenic with Rh2 in R1-6, viewed with white light (**D**), 520 nm light (**E**), and 520 nm light darkened with 436 nm light (**F**). Transgenic with Rh3 viewed with white light (**G**), 480 nm light (**H**), and 480 nm light darkened with 350 nm light. Transgenic with Rh4 viewed with white light (**J**), 480 nm light (**K**), and 480 nm light darkened with 376 nm light (**L**). Transgenic with Rh5 viewed with white light (**M**), 505 nm light (**N**), and 505 nm light darkened with 405 nm (**O**). Transgenic with Rh6 viewed with white light (**P**), and with 524 nm light (**Q**).

are consistent with this information.

Figure 11 presents confocal images from typical white eyed flies with GFP expression in R1-6. On the top is the deep pseudopupil, while, on the bottom, individual rhabdomeres are imaged using optical neutralization of the cornea. When compared with the corresponding images for autofluorescence (Figure 7, deep pseudopupil in top, left; Figure 6, optical neutralization of the cornea at bottom), the images show less clarity. This is a confirmation of the finding presented above (in the context of Figure 10) that GFP fills cell bodies and rhabdomeres.

Figure 12 shows GFP driven by the Rh4 promoter. In the deep pseudopupil (top) and in both views of individual rhabdomere tips after optically neutralizing the cornea (bottom), the R7 rhabdomere and the cell body fluoresce. Some rhabdomeres are bright, presumably expressing Rh4, while others (the rhabdomeres that have Rh3) are dim. Analysis of multiple serial planes gives counts of 426 positives, 782 negatives, 256 unknowns, and 183 questionable positives.

Figure 13 shows GFP driven by the Rh3 promoter and targeted to the rhabdomere by attachment to Rh3. In contrast with the situation for Rh4, fluorescence is restricted to the rhabdomere as expected. Analysis of Rh3-Rh3-GFP is equivocal: most R7's fluoresce (330 positive to 177 negative), but only a small percentage (about 30%) were strikingly positive.

DISCUSSION

Transgenic Drosophila *with ectopic rhodopsins: Drosophila* stocks with Rh2-Rh6 replacing Rh1 are extremely useful. An interesting use of the Rh(1+4) stock was in studies of calcium's role in phototransduction. Green light (543.5 nm) was used to excite Ca^{2+}-orange, a calcium indicator for monitoring. UV stimulation of UV "replaced" R1-6 receptors insured sufficient wavelength separation so that rhodopsin activation and

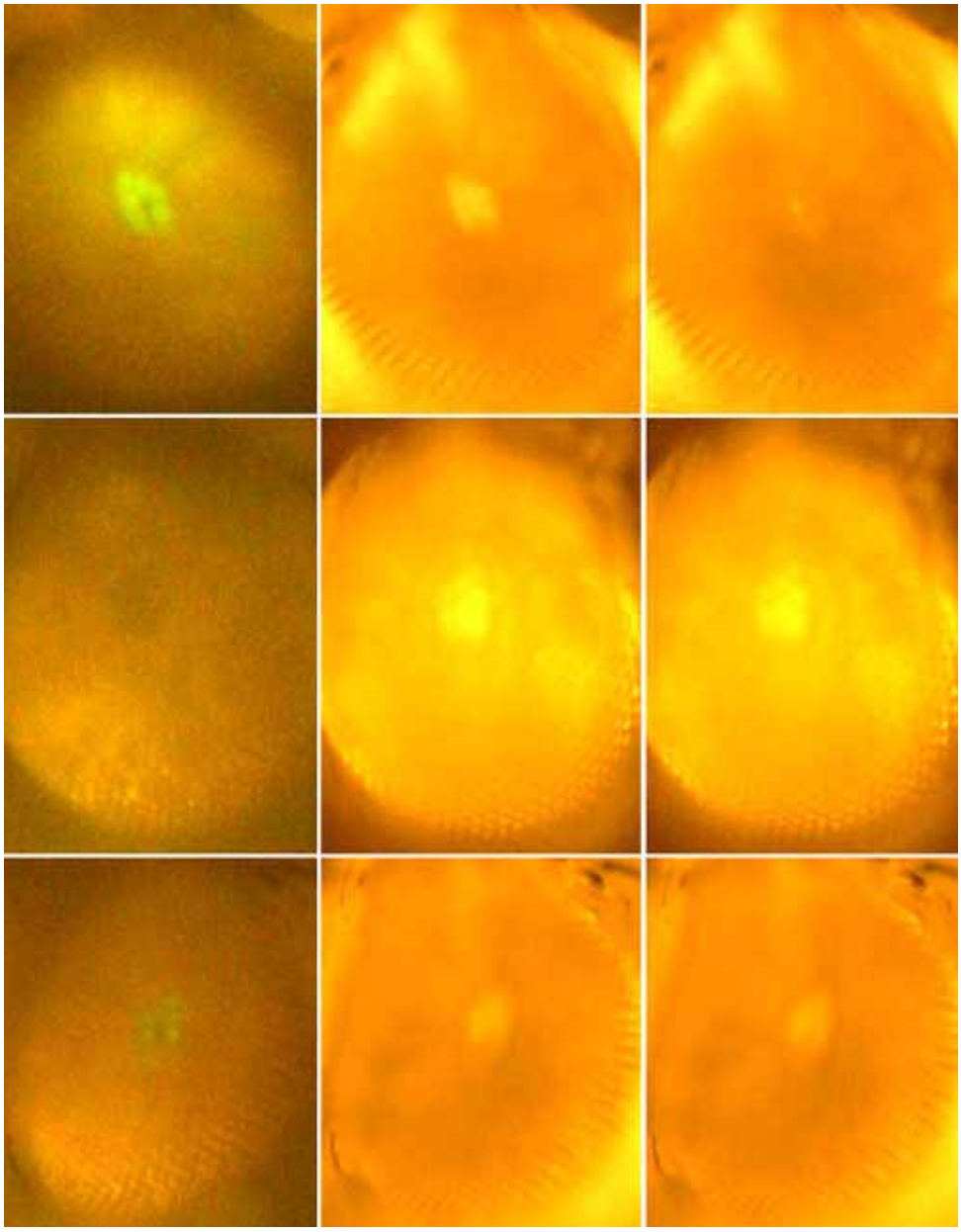

Figure 10. R1-6 GFP (standard fluorescence microscope) and rhodopsin conversions (light microscopy). Deep pseudopupils of typical white eyed fly from the cross that gives GFP expression in R1-6. Regular (vitamin A replete) food (top row). Vitamin A deprived (middle row). Yeast food (bottom row). Fluorescence excited by 480 nm light (left), transmission of 579 nm light (middle), and transmission of 579 nm light with 480 nm actinic stimulation (right).

calcium monitoring did not confound each other [35]. Our use of these stocks pursues the strategy of their developers [11,15,21,22], to utilize expression of minor rhodopsins in the fly's predominant photoreceptor type, R1-6, to investigate receptor spectral mechanisms.

Confocal microscopy of opsin autofluorescence does not distinguish bright from dim R7/8 autofluorescences: Confocal microscopy [19,36] provides higher resolution than conventional fluorescence [29,37,38] especially for resolving different R7 types [19]. Bright and dim R7/8 rhabdomere tips are not mediated by R7 opsins since neither Rh3 nor Rh4 fluoresce (Figure 7). R8 makes up the proximal 1/3 of the R7/8 rhabdomere, with Rh5 paired with Rh3 and Rh6 paired with Rh4 [15,16,22]. R8 opsins are also not responsible for bright versus dim R7/8 rhabdomere tips since Rh5 is only slightly brighter than Rh6. In summary, autofluorescence of Rh3, Rh4, Rh5, and Rh6 did not answer which R7 has bright versus dim autofluorescence.

GFP analysis does distinguish bright from dim R7/8 autofluorescences: Our analyses using GFP suggest that the R7 cells expressing Rh4 are those that autofluorescence brightly. When Rh3's promoter drove Rh3-GFP, most R7s fluoresce, but only a small percentage (about 30%) were strikingly positive. These 30% strikingly bright rhabdomeres certainly express Rh3; we argue that the others are showing autofluorescence and contain Rh4. Alternatively, the Rh3 promoter may not be completely selective [23], leading to the same conclusion. Previous work suggested that the ratio of Rh4:Rh3 is 2:1 [11]. Based on our counts, we put the ratio at 2.42:1.

GFP reports transcriptional control of opsin's reporter without interfering with receptor function: GFP serves as a reporter for transcriptional control of the opsin promoter by retinoids, as implied by our comparison of flies reared on vitamin A replete and deficient diets. The fact that rhodopsin-

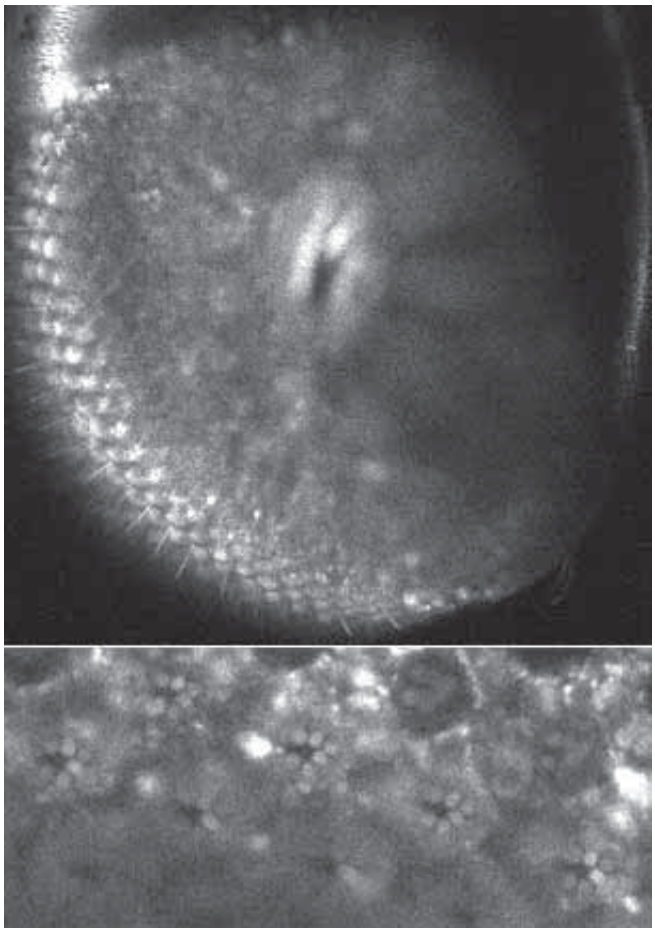

Figure 11. R1-6 GFP (confocal microscopy). Deep pseudopupil (top) and optical neutralization of the cornea (bottom) of typical white eyed fly from the cross that gives GFP expression in R1-6.

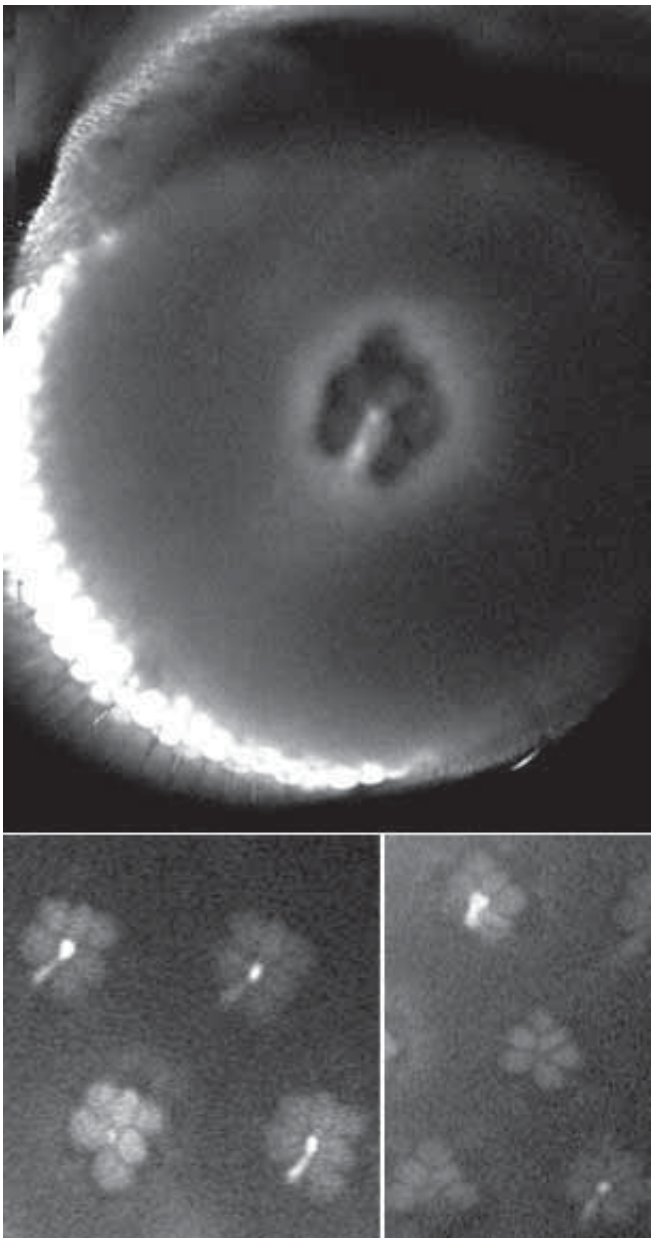

Figure 12. Green fluorescent protein labeling for Rh4 promoter driven R7s. Rh4-GFP (Rh4's promoter driving GFP into R7). Deep pseudopupil (top) and two samples of individual rhabdomere tips (optical neutralization of the cornea; bottom).

metarhodopsin conversions can still be observed when R1-6 are flooded with GFP proves that GFP does not interfere with the photochemistry of visual pigments. Further work (unpublished) shows that GFP does not interfere with receptor function as measured by the electroretinogram (ERG), even when the flies are aged several weeks. Specifically, the ERG shows that (1) flies with GFP are fully sensitive; (2) waveform is not altered (i.e., on-and-off transients and receptor potential are normal); and (3) a prolonged depolarizing afterpotential (PDA) is elicited by blue light and repolarized by yellow light. The convenience of studying the pseudopupil in live flies and the demonstration that cells survive the presence of GFP, should facilitate further promoter-reporter analyses.

The fluorophores responsible for rhabdomere autofluorescence: A complicated literature unfolded concerning R1-6 fluorescence. Summarizing, (1) the metarhodopsin from R1-6's rhodopsin (Rh1) fluoresces [39]; (2) a retinoid that sensitizes R1-6's blue rhodopsin (Rh1) to UV also contributes [28]; (3) a product of very intense UV and blue stimulation in R1-6 called M' fluoresces [27,28,39]; and (4) accessory pigments (a blue blocking carotenoid and UV sensitizing pigments) were found in the brightly fluorescing R7/8 receptors (R7y) in other genera [38]. Which of these four possible sources determines the relative emissions of all 6 rhodopsins (Rh1=Rh5>Rh6>Rh2>Rh4>Rh3) is unclear:

(1) Rh2 to Rh6 and/or their corresponding metarhodopsins may have less fluorescence than Rh1's metarhodopsin.

(2) Although Rh2 [21] and Rh5 [22] are coupled to R1-6's UV sensitizing retinoid when expressed in R1-6, the sensitization of Rh3, Rh4, and Rh6 is not known. This is an important consideration since bright R7 rhabdomere tips are substantially brighter (Figure 6) than expected from Rh4 plus Rh6. Accessory pigments are important, especially in R7 [40].

(3) Fluorescence and confocal microscopy utilize lot of light, enough to create M', but we found that blue light increased emission only for Rh1 and Rh5 [41]. Bright light products (M') may contribute to autofluorescence, but only for Rh1 and Rh5 (the brightest ones).

(4) If *Drosophila* have the accessory pigments of R7y and R8y [38], (and that is uncertain), it is not known whether these R7y and R8y pigments are co-expressed when Rh4 or Rh6, respectively, are ectopically expressed in R1-6.

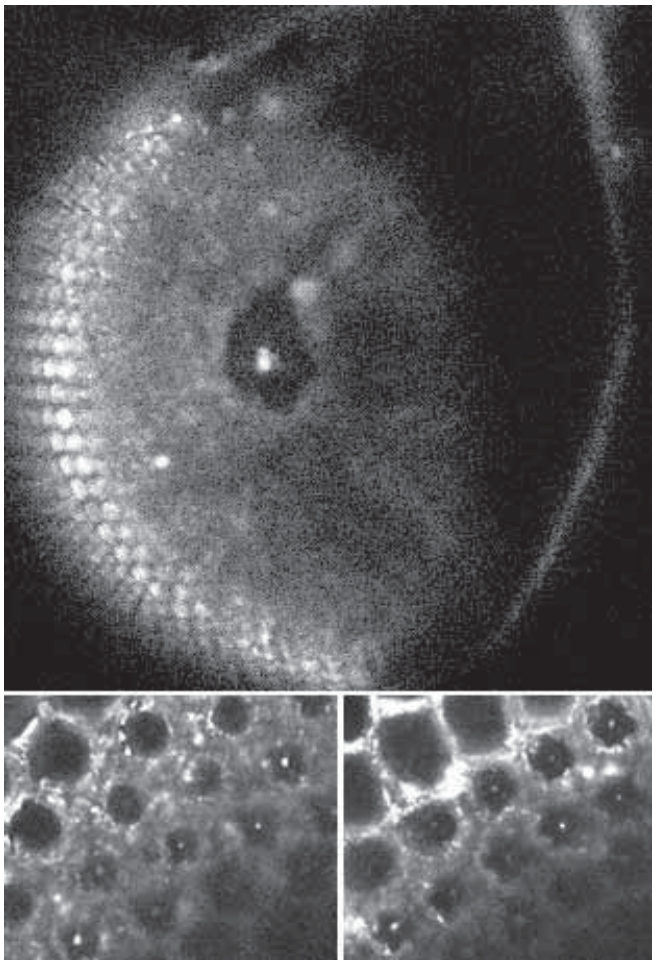

Figure 13. Green fluorescent protein labeling for Rh3 promoter driven R7s. Rh3-Rh3-GFP (Rh3's promoter driving GFP attached to opsin, Rh3, into R7). Deep pseudopupil (top) and two samples of individual rhabdomere tips (optical neutralization of the cornea; bottom).

Visualizing rhodopsin: In 1942, G. L. Walls [42] wrote, "With the very sloppiest of technique, we can mount the fresh dark adapted retina of a frog or a goldfish on the microscope and still see the rich wine of rhodopsin filling its rods" cited by Liebman [43]. Here, we show, for the first time, the red color of the "visual pigment" in the fly eye. By "visual pigment," we mean rhodopsin plus metarhodopsin plus any accessory (sensitizing and/or blocking) pigments. Rh1 and Rh6, and, to a lesser extent, Rh5 look pink relative to R7/8 which contain a combination of Rh3, Rh4, Rh5, and Rh6 in the deep pseudopupil. This is explained by rhodopsin plus metarhodopsin absorbing considerably at short and middle visible wavelengths.

Rhodopsin-metarhodopsin conversions: In 1978, Lo and Pak [44] observed that blue light decreased the transmittance through R1-6 rhabdomeres in the deep pseudopupil. Their interpretation, namely that they were somehow visualizing the state of depolarization of the receptors, created controversy, especially since Stavenga and coworkers [45] had attributed measured transmission differences to rhodopsin-metarhodopsin conversions five years earlier. Stavenga was eager to present a photograph dramatizing the visualization of what he had measured quantitatively, and so he published a photograph of this laboratory's preliminary work [46], work that was improved by the time this laboratory's full paper was published [26]. In the decades since then, quantifying and viewing rhodopsin-metarhodopsin conversions has been useful in countless studies. The characterization of five other rhodopsins in *Drosophila* and the convenience of digital color photography, prompted us to re-examine conversions. For Rh1-Rh5, the findings were straightforward, explained in terms of what was known about the respective wavelengths of the pig-

ments. Despite the lack of surprises, the demonstration is useful. For instance, when only a red eyed Rh(1+3) stock was available, we viewed the pseudopupils in live flies that were anesthetized but not fixed to a microscope slide, and, by so making the UV rhodopsin of the photoreceptors a visible trait, we constructed *w;Rh(1+3)* with a genetic cross.

The story for Rh6 is very interesting. (1) It is not closely related to the five other *Drosophila* rhodopsins; (2) It is the only one of six with a shorter wavelength metarhodopsin (R-508 nm, M-468 nm); and (3) The apparent metarhodopsin is less thermally stable [22]. Also, *sev* (*sev*enless) flies have Rh6 but not Rh5 [18], suggesting that all conclusions about R8 from this laboratory's work, based on using *sev* [1], applies to Rh6, not Rh5. At that time [1], it was noted that R8 was "non-adapting," since we could not isolate rhodopsin from metarhodopsin by chromatic adaptation. This is confirmed in *wRh(1+6)* flies here: Although R1-6 are bright and slightly pink when viewed with white light, R1-6 remain continuously dark when transilluminated with 514 nm light (even without additional blue adaptation to convert metarhodopsin to green absorbing rhodopsin). The present findings suggest that the rhodopsin and metarhodopsin are not chromatically separable despite the suggestion by Salcedo et al. [22] that rhodopsin peaks at 508 nm while metarhodopsin peaks at 468 nm.

Retinal multiplicity in Drosophila *compared to* Musca*:*

There remains a major discrepancy in the *Musca* and *Drosophila* literatures. Although R7y and R7p in *Musca* are both UV receptors, R7y has a blue absorbing rhodopsin, a UV sensitizing retinoid, and a blue blocking carotenoid [38]. In *Drosophila*, by contrast, two R7 UV rhodopsins, Rh3 and Rh4, were found [11]. Based on ratios of subtypes and slight differences in UV spectral sensitivity, Rh3 and Rh4 were thought to correspond to R7p and R7y, respectively [11]. Our GFP analysis confirms this conclusion, assuming that p (pale) and y (yellow) for Musca correspond to dim and bright for Drosophila. However, the discrepancy of a UV Rh4 in *Drosophila* and a blue opsin in *Musca* R7y has not been addressed.

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