A new visualization approach for identifying mutations that affect differentiation and organization of the Drosophila ommatidia

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SUMMARY

The Drosophila eye is widely used as a model system to study neuronal differentiation, survival and axon projection. Photoreceptor differentiation starts with the specification of a founder cell R8, which sequentially recruits other photoreceptor neurons to the ommatidium. The eight photoreceptors that compose each ommatidium exist in two chiral forms organized along two axes of symmetry and this pattern represents a paradigm to study tissue polarity. We have developed a method of fluoroscopy to visualize the different types of photoreceptors and the organization of the ommatidia in living animals. This allowed us to perform an F1 genetic screen to isolate mutants affecting photoreceptor differentiation, survival or planar polarity. We illustrate the power of this detection system using known genetic backgrounds and new mutations that affect ommatidial differentiation, morphology or chirality.

Key words: Drosophila eye, Ommatidia, Rhodopsin, Planar polarity, Genetic screen

INTRODUCTION

In the past decade, Drosophila has proven to be a very powerful species with which to address a broad and still increasing range of developmental and cell biology issues. The Drosophila eye has provided an exquisitely accessible system to study processes ranging from cell proliferation to neuronal differentiation and to apoptosis (for reviews, see Treisman, 1999; Fortini and Bonini, 2000). The eye of Drosophila is composed of approximately 800 basal units called ommatidia. Each ommatidium contains eight photoreceptor (PR) neurons (R1-R8) organized in a stereotypical trapezoid (Figs 1A,B, 2B), as well as 12 accessory cells (i.e. pigment, cone and bristle cells) (Ready et al., 1976; Wolff and Ready, 1991). During eye development, PR specification occurs sequentially. The founder cell R8 plays a central role in the formation of a cluster of five cells (Ready et al., 1976; Harris et al., 1976; Tomlinson, 1985; Wolff and Ready, 1991). It specifies the R2 and R5 PRs, and then the R3-R4 pair. Subsequently, after a last round of cell division, R1 and R6, and, finally, R7 are recruited and begin differentiating within the newly formed ommatidium (Cagan, 1993; Kumar and Moses, 1997; Brennan and Moses, 2000). Finally, 12 accessory cells are recruited to form the adult ommatidium, which is endowed with six outer PRs (R1-R6) that surround the two inner PRs – R7 placed on top of R8 along the distal-proximal axis of the retina (Fig. 1B).

A very puzzling problem in developmental cell biology is how a cell achieves its correct positioning relative to its neighbors. In many developing tissues, a certain degree of relative or absolute cell polarization must be achieved. Most often, a particular cell type or a group of cells is patterned probably through a network of signaling molecules. This implies that these cells are able to ‘read’ the relative concentration of different patterning molecules in order to extract spatial clues. In turn, those cells respond in terms of positioning or polarization within the differentiating field of cells. One of the most striking examples to illustrate this problem is the establishment of planar polarity in the developing eye of Drosophila. In addition to the regular arrangement of PRs within each ommatidium, the fly retina is organized into two compartments that contain symmetrically organized mirror-image ommatidia (Dietrich, 1909; Fig. 2B). The midline region of the eye or ‘equator’ is a frontier from which a signal emanates that polarizes the ommatidia in two chiral forms, with the dorsal and ventral ommatidia ‘pointing’ in opposite directions (Wehrli and Tomlinson, 1995; Reifegerste and Moses, 1999; Mlodzik, 1999; Strutt and Strutt, 1999). In addition, an antero-posterior asymmetry also exists in the eye. For instance, the chiral trapezoid formed by the outer PRs R1-6 always presents the same side (R1, 2 and 3) to the anterior of the head. This antero-posterior asymmetry is likely to be tightly linked to the imprint provided by the progression of the morphogenetic furrow (Chanut and Heberlein, 1995; Strutt and Mlodzik, 1995; Wehrli and Tomlinson, 1998).

The outer PRs R1-R6 are the functional equivalent of the
rod PRs found in mammals. They contain Rhodopsin I (Rh1), an opsin presenting a broad absorption spectrum with a maximum in green light ($\lambda_{max}$, approx. 490 nm; Ostroy et al., 1974; Hardie, 1979). The gene ninaE, which encodes Rh1, is expressed in all 6 outer PRs (O’Tousa et al., 1985; Zuker et al., 1985). In flies, Rh1 is associated with a second, UV-sensitizing chromophore ($\lambda_{max}$, approx. 360 nm; Hardie, 1985; Kirschfeld and Franceschini, 1977) that broadens the spectrum of light detection of the outer PRs. Color discrimination is presumably achieved by the inner PRs R7 and R8 that are the functional equivalent of the vertebrate cones (for review see Pichaud et al., 1999). R7 and R8 contain different rhodopsins and compare their inputs in the medulla part and higher regions of the optic lobe. In fact, there are two classes of ommatidia that contain different complements of rhodopsins in their inner PRs: pale ommatidia (see below) contain Rh3 in R7 and Rh5 in R8 while yellow ommatidia contain Rh4 in R7 and Rh6 in R8 (Franceschini et al., 1981a; Hardie, 1979; Hardie, 1985; Chou et al., 1996; Papatsenko et al., 1997). These two types of ommatidia, which are distributed stochastically in the retina, presumably expand the spectrum of colors that can be resolved by the eye.

While the molecular events underlying patterning, differentiation and specification of the adult eye have been an active area of research, many questions remain unanswered. A major technical difficulty with many of these genetic studies is the fact that the current methods of analysis require the sacrifice of the animal, thus only allowing realization of second generation (F2) screens for mutations affecting these processes. Although first generation (F1) screens have been developed based on the external morphology of the eye, this approach has predominantly been limited to dominant modifier (enhancer or suppressor) screens of a given mutation (Karim et al., 1996). Most often these screens have used the roughening of the eye as read-out. Such an approach requires that the mutation to be identified interact in a dominant fashion with the particular genetic situation created for the screen to disrupt ommatidial cell stacking leading to the rough aspect of the eye.

Here, we have adapted and developed a technique of ommatidial fundus fluoroscopy originally described to assay the spectral properties of opsins in the large eye of white mutant Musca flies (Franceschini, 1983; Franceschini and Kirschfeld, 1971a; Franceschini and Kirschfeld, 1971b). We used both the auto-fluorescent properties of visual pigments as well as the fluorescence of the green fluorescent protein (eGFP) introduced into PRs to visualize individual cells in adult eyes of living Drosophila flies. We illustrate the use of this powerful technique to assay a set of characteristics of the eyes such as planar polarity, ommatidium cell content, PR differentiation and cell morphology. These parameters were previously available only through complex histological methods. In addition, we present a large-scale F1 recessive mosaic screen using this non-invasive technique to identify genes that affect these characters in mutant homozygous clones. We illustrate this type of mutants with examples isolated in our screen. All documents presented in this report (except for Fig. 4F) were realized in living flies and thus represent a novel system to examine a wide variety of cellular processes in the Drosophila eye.

**MATERIALS AND METHODS**

**Fly stocks**

Ey-flip: FRT80, P(w+) and FRT82, P(w+) flies used for the screening procedure were provided by Barry Dickson and Jessica Treisman (Newsome et al., 2000; Benlali et al., 2000). The yw; mwh iro-C(DM)F3 FRT80A/TM3 was used in the iro-C mosaic experiments (Cavodeassi et al., 1999). sev-fz, sev-dsh and sev-N(ecd) have been previously described (Fortini et al., 1993; Fanto and Mlodzik, 1999; Tomlinson and Struhl, 1999) and were used to create symmetric ommatidia.

**Fig. 1.** Ommatidia characteristics.

(A) Lens facets observed by epifluorescence using green light illumination. The white circles indicate the position of bristle cells. Under these conditions of observation, the highly ordered honeycomb structure of the eye appears as well as the accessory pigment cells. (B) Close up showing the different cells that compose an ommatidium (principally pigment cells and lens facet), with the interpretation using a schematic diagram adapted from Wolff and Ready of a proximo-distal cross section of an ommatidium (Wolff and Ready, 1993). (C) Mosaic eye composed of red (white+) and white (white mutant) tissue. The absence of pigment in the mutant tissue leads the pigment cells to be invisible. Asterisks indicate mosaic ommatidia for which only one of the pigment cells is not detected. (D) A ‘rough’ eye as seen under green light illumination. This imaging reveals the chaotic aspects of a mutant eye obtained in our EMS screen. An asterisk indicates two fused lens facets (lacking a pigment cell), and an arrowhead points to a square lens facet, characteristic of missing accessory cells.
eGFP transgenes

prh1-eGFP is a direct fusion between a minimal rh1 promoter (genomic –252 +18 fragment) and eGFP (Clontech). prh1 is a blunted KpnI fragment cloned in a CHABXαal containing a SV40 poly A (Wimmer et al., 1997) and eGFP (BamHI/Xbal). prh4-eGFP is a direct fusion of a minimal rh4 promoter (~373 + 85) and eGFP in CHABXαal. prh4 is an EcoRI/BamHI inserted link to the BamHI/Xbal eGFP. prh3-Rh3-eGFP is a fusion between a PCR amplified fragment of rh3 genomic DNA and eGFP. Primers used here amplified the rh3 loci from ~243 to the end of the transcript, while replacing the stop codon by a junction sequence: PRVAT. The PCR primers were designed so that an EcoRI site was created 5’ to and an AgeI 3’ to the rh3 gene PCR product. These sites were used to fuse prh3-Rh3 in frame with the eGFP in CHABXαal. The Rh5-eGFP fusion protein was realized in PUAST after PCR amplification of the Rh5 open reading frame (ORF) using primers so that an EcoRII and an AgeI site were inserted in 5’ and 3’ of the ORF. The same junction peptide as used for prh3-Rh3-eGFP was used here. These restriction sites were then used to fuse Rh5 ORF and the eGFP in frame in CHABXαal. Fusions between Rh1, Rh4, Rh6 and eGFP were realized both under the control of their respective promoters in CHABXαal and in PUAST (Brand and Perrimon, 1993). The respective rhodopsin ORFs were PCR-amplified and fused in frame to eGFP. In the case of Rh1-eGFP, the junction peptide between the rh1 and eGFP was RVAT. For each of these constructs, we established transgenic lines on each chromosome (X, II and III). The rh1, rh3 and rh4 promoters were obtained from C. Zucker’s lab (Mismar and Rubin, 1987; Fortini and Rubin, 1990).

Sample preparation

In order to examine the flies under water immersion, we fixed them on the surface of a 2% agarose gel poured on the surface of warm agarose (40-42°C) and were maintained under a flux of CO2 was pulsed on the surface of the agarose. Once the agarose was solidified, the flies were covered with cool (approx. 15°C) water that permits the observation under immersion and also maintains the effect of the anesthetic.

During imaging, the heads of the flies were manipulated under water using a very thin camel-hair brush, under a 10x lens (plan, 0.25 DIC). After analysis, the flies of interest were eventually detached from the agarose and collected at the surface of the water that permits the observation under immersion and also maintains the effect of the anesthetic. The flies required more than 30 minutes to recover. However, we did not observe significant effects of this procedure on the lifespan or fertility of the rescued flies (data not shown).

Imaging of photoreceptors

We have designed a very simple Plexiglas movable stage that is able to hold the cover of a 90 mm diameter Petri dish in its center. In this set up, the fly preparation (in a 50 mm Petri dish) was placed in the center of the plastic stage, allowing us to move the samples along the normal axes of an epi-fluorescent microscope. We used a Nikon Microphot-SA that has an aperture stop that provides an important advantage (although it is not essential) as it allows a better signal-to-noise ratio by adjusting the light to the acceptance angle of the PRs rhabdomeres. A super high-pressure mercury lamp (Hg 100 watts) was used as light source (USHIO Electric). UV illumination of the flies was performed in a narrow bandwidth (excitation (ex), 365 nm) of mercury spectrum with a filter (Dichroic Mirror (DM), 400; Barrier Filter (BA), 400 nm; Nikon). Detection of the photo-conversion of Rh1 into excited states (M/M’) was performed using a band-pass FITC-HQ: filter (Nikon) (ex, 480/40 nm; DM, 505; BA, 535/50 nm). For the visualization of M/M’, we used a band-pass Texas Red cube HQ:TR filter (Nikon) (ex, 540-580 nm; DM, 595; BA, 660-660 nm). Detection of the yellow ommatidia was performed in red eye flies (wild type) using the technique of aperture stop (Franceschini and Kirschfeld, 1971) and a band-pass FITC HQ:F filter. The aperture was closed so that the transmitted light was narrowed down to the directivity angle of the rhabdomere of the PR. Examination of flies was performed using a 20x water immersion objective with a 0.4 optical aperture (Nikon). Images were captured using a digital camera (Spot-2; Diagnostic Instrument).

Reflecting imaging was performed using a directional source of visible white light (60 W halogen bulb). This light source was placed so that the immersed fly preparation was illuminated by white light. We used alternatively a 10x (0.25) and 20x (0.4) objectives, both of which present a reasonable working distance, so that the sample could be illuminated. In this case, water allowed the homogenous diffusion of the light source.

F1 genetic screen

We used EMS as a mutagen and fed 3-day-old males according to previously described procedures (Ashburner, 1989). The FRT mutagenized males were crossed with virgin females carrying the eye-specific source of flip recombinase ey-flp (Newsome et al., 2000; Benlali et al., 2000) at a 1:1 ratio. The direct progeny of these flies exhibited large white mutant clones in an otherwise red eye background (see Figs 3A, 6). Males and females were then separated and kept for 7 days in vials. We introduced this delay between fly hatching and screening in order to allow eventual photoreceptor degeneration to occur in the mutant clones. Each interesting mutant was rescued as described above and crossed to flies carrying appropriate balancer chromosomes to establish a stock.

RESULTS

Optical neutralization of the cornea

When illuminating a fly eye with blue or UV light, the deep pseudopupil (PP) can be observed using a low power microscope (10x) equipped with a large optical aperture objective. The PP is detected when focused in the depth of the eye where the center of curvature of the eye is reached (approx. 180 µm in Drosophila) (Franceschini and Kirschfeld, 1971b; Fig. 2A). It originates from the superposition of the virtual images of a number (n) of (identical) rhabdomeres, with n being defined by the optical aperture of the objective (Franceschini, 1972). In our assay (10x; optical aperture, 0.25) we determined n to be approx. 40 ommatidia by counting the number of illuminated facets. The formation of the PP is rooted in the geometrical organization of the eye with the value of the inter-photoreceptor and inter-ommatidial angles being identical (Franceschini and Kirschfeld, 1976; Kirschfeld and Franceschini, 1968). In practice, the PP can be seen as a magnification of a single ommatidium since each of the six dots seen in Fig. 2A is about 15 µm in diameter (approx. 10x magnification when compared with the diameter of an individual rhabdomere). The PP represents a very useful tool to assay the integrity of all PRs within the ommatidia. However, it is not suitable for the detection of alterations in a single ommatidium.

To be able to visualize PR fluorescence in the Drosophila retina at the level of individual ommatidia, we have adapted the technique of neutralization of the cornea (Franceschini
and Kirschfeld, 1971a; Franceschini et al., 1981a). The original method consists of placing a thin film of a transparent medium on the surface of the eye that has a similar refractive index to the corneal lenses (such as water, oil, or nail polish). This optically neutralizes the air/cornea convergent dioptric system (Franceschini and Kirschfeld, 1971a). It is thus possible to visualize images of the most distal tips of the individual rhabdomeres, a set of highly specialized cell membranes organized into microvilli that concentrate and present opsins in the path of light. To efficiently neutralize the refraction of the cornea, we chose to immerse the entire fly in water. Even after water immersion for up to 2 hours, we found the flies to be perfectly healthy and fertile. To maintain the flies under water for observation, we attached them to the surface of a 2% agarose gel (see Materials and Methods). We attach anesthetized flies by depositing them on the surface of 40°C agarose bed poured in the cover of a 50 mm Petri dish. After hardening of the agarose, the living flies are covered with cold water and observed using the appropriate optical conditions (see below, Materials and Methods, and Fig. 5A).

**External morphology of the eye**

While using water immersion to look at PRs, we realized that we could obtain a very precise description of the morphology of the eye by using different sets of optical filters. We found that using green light illumination (Texas Red filter; Barrier filter, 600-660 nm) we could very precisely assay the integrity of an eye, i.e. its content in pigment cells and the hexagonal shape of the individual facets. In these conditions, we were able to visualize very accurately the individual pigment cells (secondary and tertiary) in living flies when focused on the plane of the eye surface (Fig. 1A,B). As these cells support the hexagonal structure of the lens facet, defects or missing pigment cells lead to abnormalities in the crystal-like organization of the compound eye. In our assay, very subtle defects in cell stacking could be detected. These defects ranged from squared facets that lead to incorrect cell stacking, or fusion of ommatidia (Fig. 1D). Furthermore, in case of mosaicism in the eye, where clonal tissue is marked by the absence of pigments caused by a white mutation, this technique allows the detection at the single cell level of mutant ommatidia in an otherwise wild-type background (Fig. 1C).

**In vivo detection of outer photoreceptors**

Previous studies have reported the visualization of the outer PRs in white mutant eyes of living Musca domestica flies, based on the autofluorescence of their pigments (rhodopsin and accessory pigment) (Franceschini et al., 1981a). In addition, two subtypes of ommatidia could be detected in these large eyes, owing to different spectral properties of the R7 inner PRs (Franceschini et al., 1981a).

We were able to detect the outer PRs (Fig. 2) and the two subclasses of inner Drosophila PRs, in both white mutant and wild type Drosophila eyes (Fig. 2B) using epifluorescence microscopy and commercially available optical filters (see Materials and Methods). By focusing slightly below the surface of the cornea (below the pseudo-cone), we could obtain an image of the rhabdomere tips. We used UV illumination to visualize the outer PRs through the pinkish/white fluorescence of the UV-sensitizing accessory pigment found in R1-R6 (Kirschfeld and Franceschini, 1977; Fig. 2A,B). In this case, the signal was sustained for only a few seconds (approx. 10 seconds) but could be regenerated after a few seconds of green light illumination (band-pass Texas Red filter; BA, 600-660 nm). Therefore, using alternating exposure to blue/green and UV light, we were able to visualize, and thus qualitatively and quantitatively assay parameters such as planar polarity, rhabdomere morphology or the number of PRs in each ommatidium.

To complement this detection system, we also used blue light excitation of Rh1 in the six outer PRs. The red fluorescence obtained under these conditions (Fig. 2C) is due to the conversion of Rh1 into a derivative form of metarhodopsin 1 (Rh1M) referred to as M’ (λ<sub>max</sub>, approx. 660 nm) (Kirschfeld and Franceschini, 1977), an excited state of Rh1. For this experiment, we used blue light (band-pass FITC-HQ-F filter) for approx. 5 seconds, and visualized the red fluorescent M/M’ using a band-pass Texas Red filter (Fig. 2C). M/M’ is deactivated within a few seconds in Drosophila and the presence of pigments in wild type eyes renders M/M’ fluorescence detection more difficult than the more contrasted method using the UV sensitizing pigment. Therefore, the
autofluorescence of M/M' is more labile than the previous assay based on the autofluorescence of the UV-sensitizing pigment. However, as the regeneration of the UV-signal (Fig. 2B) requires blue/green light illumination, we can use a 'ping-pong' system that allows to alternatively visualize the outer PRs under UV light, then to visualize the same rhabdomeres as we regenerate the UV signal under green light. Thus, these two detection systems can be seen as a single one, with one being necessary to regenerate the other.

These two experimental conditions involve epifluorescence microscopy. In these conditions, red tissue appears dark, while white mutant tissue appears greenish due to the autofluorescence of the cornea. In addition, we also used white light illumination to visualize both the eye color and the PRs. This can be very useful in order to be able to detect both the eye color (white versus orange versus red) and the PRs in the same preparation, situations commonly encountered in mosaic experiments, for example. We used a 60 W halogen directional lamp placed beside the microscope to perform an illumination of the eye, and observed with either a 10× (optical aperture, 0.25) or a 20× (optical aperture, 0.4) water immersion objective, which both present a comfortable working distance. This approach is different from the previously described antidromic illumination of the eye, which involves a light source placed underneath the fly head so that light shines through the head/eye PRs of the fly (Franceschini and Kirschfeld, 1971a; Hardie, 1985; Jackson, et al., 1998). In our assay, the dark-autofluorescence of the PRs is due to the contrast between the light absorbing rhabdomeres and the reflected light from the rest of the tissue (Fig. 3C). This visualization is possible using a regular microscope and does not require fluorescence. Both outer and inner PRs could be visualized in living flies, and their belonging to white mutant or red wild-type tissue could be precisely assessed (Fig. 3A,B).

**In vivo detection of inner photoreceptors**

In Musca, approximately 70% of the inner PRs emit, under blue light excitation, a yellow/green fluorescence (so called yellow subtype) due to the likely presence of a photostable screening pigment in the corresponding R7 cells (Franceschini, 1983; Hardie, 1985). In the yellow subtype, this pigment is co-expressed with Rh4, a UV-sensitive opsin ($\lambda_{\text{max}}$, 375 nm) (Montell et al., 1987; Feiler et al., 1992). The remaining 30% of ommatidia, which do not fluoresce, constitute the pale subtype and contain an R7 cell expressing another UV-sensitive opsin ($\lambda_{\text{max}}$, 345 nm), Rh3 (Franceschini et al., 1981a; Franceschini et al., 1981b; Zuker et al., 1987). We could reproduce these results in *Drosophila* and easily distinguish the two types of ommatidia based on their inner PR fluorescence (not shown).

To complete the imaging based on auto-fluorescence of opsins, and to better visualize the different subtypes of PRs, we have also developed transgenes where the coding sequence of the enhanced green fluorescent protein (eGFP, Clontech) is placed under the control of individual *rh* minimal promoters (prh) (Fortini and Rubin, 1990). As the mutually exclusive expression of *rh* genes in the various types of PRs is regulated at the transcriptional level (Fortini and Rubin, 1990; this work), it can be mimicked by fairly short promoters attached to reporter genes. We constructed several transgenes to mark the outer or inner PRs.

For outer PRs, we used the *rh1* promoter (Mismer and Rubin, 1987) fused to eGFP, which gives a strong level of expression and thus eGFP signal in R1-R6 (Fig. 4B). Using prh1-eGFP, we were able to detect a very bright DPP (data not shown; Mollereau et al., 2000) and, after neutralization of the cornea, every single outer PR could clearly be seen under blue light illumination (Fig. 4B,C). The image of large fields of the retina that contain >50 ommatidia could be observed, which allows a very precise evaluation of the shape of individual ommatidia, their orientation and the location of the equator. This detection system has also the advantage that it can be sustained for a longer time than the auto-fluorescence of opsin or accessory pigments. In addition, using horizontal sectioning of the eye, the axons of the corresponding PRs could be traced all the way to the neuropile in the lamina part of the optic lobe by looking at eGFP (data not shown).

For inner PRs, we used different types of transgenes: for pale R7 cells (which contain Rh3), the transgene consisted of the *rh3* (~343 +18) promoter (Zuker et al., 1987) driving expression of the Rh3 coding sequence fused in frame at its

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**Fig. 3.** Imaging of PRs through reflective microscopy. (A) Mosaic eye composed of red (wild-type) and white (white) eye tissue as observed under white light from an outside source and with water immersion. Not only are the clones visible, but so are the outer PRs. The equator is represented by a white line. (B) Close up of a field of ommatidia under white light. The blue and yellow arrows represent the two chiral forms of ommatidia present in this field. A white arrowhead points to a single clonal (white) ommatidium. (C) Grayscale magnification of a single ommatidium observed in the same conditions. Note the presence of both the outer (1-6) and inner PRs (7/8).
This fusion allows eGFP to be addressed to, and concentrated in, the rhabdomeres (as shown in the horizontal section, Fig. 4F). This dramatically improves the detection limit when compared with a simple rh3 promoter fused to eGFP (data not shown). The eGFP signal obtained with a single copy of the transgene could be observed even after very long periods of observation (>30 minutes). Using UV illumination of flies carrying the prh3-Rh3-eGFP transgene, we were able to visualize simultaneously the outer PRs (through autofluorescence of the accessory pigment; Fig. 4A) and the eGFP (Fig. 4D) present in pale R7 cells (the microscope field in Fig. 4A is the same field in UV as that shown in Fig. 4D under blue light). It should be noted that while the prh3-Rh3-eGFP transgene is strongly expressed in approximately 30% of the R7 PRs in the ventral compartment of the eye (below the equator), thus precisely marking pale R7 cells (Fig. 4E), its expression is leaky with a weak expression in yellow R7 in the dorsal part (data not shown). Such dorsal pan-R7 expression was also observed with a long version (2.5 kb) of the rh3 promoter, or 3′ to its coding sequence, or post-transcriptional control.

We established a complete set of rh promoter-GFP fusion transgenes to visualize all subtypes of inner PRs, both R7 and R8. We used a direct promoter fusion of rh4 (Montell et al., 1987) (prh4-eGFP) to detect the yellow R7. The prh4-eGFP transgene was expressed in approx. 67% of R7 cells (Fig. 4G), with a stochastic distribution that precisely recapitulates the expression of rh4. The signal shown in Fig. 4G required two prh4-eGFP transgenes recombined on the same chromosome arm. This emphasizes the efficiency of the Rh3-eGFP fusion protein, which requires only a single copy for a much stronger signal. Finally, for the detection of R8 cells, we used rh5-eGFP (pale ommatidia) and rh6-eGFP (yellow ommatidia) direct promoter fusions. In both cases, the respective minimal promoters (Papatsenko et al., 1997; R. Sonneville and C. D., unpublished data) precisely marked the two subtypes of R8 cells (i.e. approx. 30% rh5 (pale ommatidia) versus approx. 70% rh6 R8 (yellow ommatidia); data not shown).

In addition, we also established a set of pUAS-Rhx-eGFP transgenes (except for Rh2 that is found exclusively in the ocelli; Pollock and Benzer, 1988) where the corresponding coding sequence of each rh was fused with eGFP behind the
Gal4 UAS promoter. Although, the C-terminal fusion of Rh3 and Rh5 to eGFP significantly improved the ability to detect strong rhabdomere signals when combined with the appropriated rhGal4 drivers, this was not the case for the Rh1, Rh4 and Rh6 eGFP fusion proteins (data not shown). These fusion proteins are expressed in the cytoplasm and thus are probably not properly folded or targeted to the rhabdomeres. One possible explanation is that the eGFP C-terminal fusion interferes with the processing of Rh1, Rh4 and Rh6 (but not Rh3 and Rh5), as has been reported in vertebrate opsins (Deretic et al., 1998). To visualize the R8 pale PRs in Fig. 4H, we used pUAS-Rh5-eGFP combined with a Gal4 driver under the control of the rh5 promoter (prh5Gal4). This combination allowed us to detect eGFP in 30% of R8 cells.

In all our experiments, we used the aperture filtering technique (Franceschini et al., 1981a; Franceschini, 1983; see Materials and Methods section). This greatly improves the signal-to-noise ratio by narrowing down the angle of the transmitted light to the value of the acceptance angle (here directivity) of the individual rhabdomeres (approx. 30°).

Evaluation of ommatidium characters: planar polarity

To test whether our detection system was appropriate to identify mutant phenotypes in planar polarity in the eye of living flies, we examined different genetic backgrounds known to affect the organization of the retina using UV-light illumination or the auto-fluorescence of Rh1 (Fig. 5C-G). It should be noted that, although the inner PRs are not visible in these conditions, the absence of R7 cells in these conditions, the absence of R7 cells in such experiments is expected, a mosaic analysis of the Iro-C genes shows that mutant clones generate ectopic equators in the dorsal compartment of the eye (Fig. 5C; Dominguez and de Celis, 1998). In these mosaic experiments, de-repression of f creates a molecular interferes with the processing of R7 cells (marked by a broken white line in Fig. 5C) and wild-type tissue (which appears dark under UV light).

The specification of R3 versus R4, which is responsible for the asymmetry of the ommatidium and the formation of two chiral forms on either side of the equator, involves the differential reception of a Wnt-like signal by Frizzled (Fz). This process is reinforced by activation of the Notch pathway in R4 through increased expression of the Notch ligand Delta (Dl) in R3. This creates a molecular and a subsequent geometrical asymmetry within each ommatidium (Cooper and Bray, 1999; Fanto and Mlodzik, 1999; Tomlinson and Struhl, 1999). Using transgenes able to activate the Wnt or Notch pathways at high and identical levels in R3 and R4 early in development, it is possible to interfere with the specification of these cells. This can be achieved by expressing fz or an
activated form of Notch (N\textsuperscript{Decd}) in R1, R3, R4, R6 and R7 under the control of the sevenless enhancer (Fortini et al., 1993; Fanto and Mlodzik, 1999; Cooper and Bray, 1999; Tomlinson and Struhl, 1999). When fz is expressed at high levels in both R3 and R4, these cells are unable to read the asymmetric Wnt-like cue that distinguishes them, and both often develop as R3 (Fig. 5D,E). However, they are sometimes able to make a stochastic choice, probably by activating the Notch pathway in one of the two cells (Fig. 5D), thus creating trapezoidal shaped ommatidia in a randomized manner. In the case of overexpression of N\textsuperscript{Decd} the vast majority of ommatidia are symmetric owing to the presence of two R4 cells (Fig. 5F,G); they also appear to have two missing outer PRs, R1 and R6 (Fortini et al., 1993; Cooper and Bray, 1999) (Fig. 5G).

It is clear that the approach presented here provides a very good alternative to tangential sectioning that is normally used to analyze these types of genetic situations: the fly is still alive after examination, allowing for a complete mapping of the retina (approx. 10 digital frames) in less than five minutes, while tangential sectioning of embedded eyes takes approximately 2 days. In the context of a genetic screen, the examination of a specimen can be performed in less than 1 minute.

An F\textsubscript{1} recessive mosaic screen to identify genes involved in ommatidium formation

The system that we describe here allows us to assay most characteristics of the eye and ommatidia in living flies, and thus permits the design of F\textsubscript{1} genetic screens to identify mutations that affect the overall cell content or morphology of the eye. To devise such a screen, we took advantage of a recently reported system that can produce mutant clones in the eye without affecting the rest of the body (Newsome et al., 2000; Benlali et al., 2000). The setup of the screen is based on the use of the yeast flipase/FRT system that allows site-specific mitotic recombination of a given chromosome arm (Golic and Lindquist, 1989; Dang and Perrimon, 1992; Xu and Rubin, 1993). In order to identify homozygous lethal mutations, this system was devised such that homozygous mutant clones are only produced in the eye of the fly through the use of an eye specific source of flip recombinase (Stowers et al., 1999; Newsome et al., 2000; Fig. 6B). In this system, the clonal mutant tissue appears white in an otherwise wild-type or heterozygous background that appears red (Fig. 6A; see also Fig. 3A). Interestingly, using blue light illumination and a regular dissecting microscope, we found that we could visualize very small clones, even individual ommatidia (Fig. 6C) that cannot be detected using white light (Fig. 6A).

Each F\textsubscript{1} progeny fly carries a single mutation that is homozygous in the large white-appearing clones. To perform the screen, the F\textsubscript{1} progeny of EMS mutagenized males is produced ‘en masse’. Butches of 50 anesthetized flies are deposited on individual clonal (white) facets detected in these blue light illumination conditions. (A,C) Pictures taken with a dissecting microscope. (D) Mosaic eye analyzed using reflective microscopy in epi-fluorescence under white light illumination. This imaging reveals both the clonal (white) and twin spot wild-type tissue (red), as well as the ommatidia organized symmetrically on either side of the equator. (E) UV light illumination of a clonal eye reveals the outer PRs in the clonal tissue and the surrounding wild-type tissue that appears dark here. A red arrowhead marks an ommatidium that is also positive for prh3-Rh3-eGFP (see F). ‘a’ marks the antenna. (F) Blue light illumination of the same clone shown in Fig. 5E. A red arrowhead points to the same ommatidium as seen under UV light in E, here positive for rh3-Rh3-eGFP.
Differentiation and organization of ommatidia

warm 2% agarose to prevent their movement and to allow water immersion of the entire fly (see Fig. 5A). The flies can stay for up to 2 hours under water without significant viability or sterility problems. For the analysis, the heads of the flies are manipulated under water using a brush that is also used to gently release the selected flies from the agarose. Selected individual mutants are used to establish balanced stocks.

To date we have analyzed approx. 30,000 mutagenized F1 flies, primarily on the third chromosome. 10,000 of those contained rh1-eGFP, which allow precise estimation of ommatidium shape, while 20,000 contained rh3-eGFP, which also allows the evaluation of rh3/rh4 ratio, but are more difficult to use for a general analysis of rhabdomere shape (which requires the use of UV light; see Fig. 2B). In this screen, we retained mutants that fell into different categories: neuroretinal degeneration, abnormal PR or ommatidial morphology, change in rh1 or rh3 expression, and planar polarity (Table 1). Most of the mutants (>70%) affecting PR morphology, differentiation or survival, as well as those affecting planar polarity are recessive lethal. Their identification was achieved by examination of the F1 living flies, under UV illumination (Fig. 6E) and under blue light (Fig. 6F). A full report of the loci identified in the different categories will be presented elsewhere. In the present work, we illustrate the validity of our approach with several representative mutants that we have isolated in the screen.

<table>
<thead>
<tr>
<th>Categories</th>
<th>Retained mutants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rh3/Rh4 ratio</td>
<td>57</td>
</tr>
<tr>
<td>Planar polarity</td>
<td>39</td>
</tr>
<tr>
<td>PR differentiation/survival</td>
<td>271</td>
</tr>
</tbody>
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Total number of flies screened = 30,000 (20,000 with rh3-eGFP and 10,000 with rh1-eGFP).

Fig. 7. Mutations that affect patterning of the eye. Sample of mutants belonging to the categories presented in Table 1. (A) Clone of mutation M80#11 on 3L. The majority of R7 express the rh3-eGFP transgene, in this focal plane. The average ratio in this mutant is 60% of rh3-eGFP positive R7 (n = approx. 2000 ommatidia). (B) Clone of tissue homozygous mutant for the gene M82#19. The opposite phenotype can be observed as the vast majority of R7 cells do not express the rh3-eGFP transgene. Only two positive R7 cells can be detected in the field (red arrowhead). This mutant presents an average ratio of 9% of rh3-eGFP-positive R7 (n = approx. 2000 ommatidia). (C) Mosaic eye showing clones (M80#A33) with no rh1-eGFP expression, indicating the absence of outer PRs. The red and yellow arrows indicate the orientations of the ommatidia. Note that the wild-type tissue is not affected (proper cell stacking and ommatidium orientation). (D) Clonal mutant tissue in gene M80#A32 is below the broken line. The orientation of the ommatidia in the wild-type tissue is indicated by red arrows. In the mutant part, the ommatidium shape is altered (e.g. see a symmetric ommatidium shown by the yellow arrowhead), and ommatidia often lack one or several PRs. (E) A clone homozygous for the M82#4 mutation (fat rhabdomeres) is surrounded by wild-type tissue. The yellow arrows indicate the polarity of the ommatidia. A red arrowhead points to a mutant ommatidium presenting abnormal rhabdomeres and missing PRs. An asterisk marks the clonal tissue. When compared with Fig. 4E, the morphology of the rhabdomeres is more affected. (F) Mosaic eye for M80#A39, a lethal mutation on 3L. Clones present ommatidia with missing PRs (or even no PRs at all). These clones non-cell autonomously reorganize the polarity of the surrounding wild-type ommatidia, thus creating new local ectopic equators. Yellow and red arrows indicates the two chiral forms of ommatidia. In all cases, the clonal tissue that is homozygous mutant appears lighter owing to the loss of the P(w+) transgene. The clones are outlined with a broken line and marked with an asterisk throughout.

Mutants affecting the ratio of Rh3 versus Rh4 in R7

We retained 57 mutants based on rh-GFP expression (Table 1) on chromosome arms 2L, 3L and 3R. Among these, 38 mutants show a significant decrease in the number of rh3-eGFP-positive R7, while the others show an increased proportion or rh3. Interestingly, the majority of the recovered mutations are homozygous viable. In addition, no significant non-cell autonomy was observed. The mutant in Fig. 7A (M80#11, semi lethal on 3L) illustrates a category of mutants showing an increased number of rh3-eGFP-positive R7 cells with over 60% positive rh3-eGFP R7 cells (n = approx. 2000 ommatidia).

The mutant presented on Fig. 7B (M82#19) shows a
significant reduction in its proportion of R7 expressing rh3-eGFP (approx. 9%, n = approx. 2000 ommatidia). We found another gene with a similar phenotype (M82#37) that fails to complement the phenotype of M82#19. It is likely to be an allele of the same gene as the two genes map to the same region of chromosome 3R and are both homozygous viable.

Mutants affecting the survival of PRs
The mutant presented in Fig. 7C shows small clones that lack PRs, while the surrounding wild-type tissue is not significantly affected as the facet lens and cell stacking seem otherwise correct. This aspect could be assessed by focusing at the surface of the lens using green light, as shown in Fig. 1. In addition, Elav staining in mosaic discs revealed no defects in the first steps of PR differentiation in clones (not shown). Therefore, it is likely that PR development is affected later, probably during pupation. Such mutation could lead to PR death in pupation or to major defects in rhabdomere formation. We have isolated 271 mutants exhibiting such phenotype and preliminary phenotypic characterization has confirmed that the vast majority of these mutants affect fairly late step of the development of PRs. This is what we expected, as we focused only on mutants that showed a wild-type aspect of the clonal tissue at the surface of the eye, but presented defects in PRs after neutralization of the cornea.

Mutants affecting rhabdomere development
Fig. 7D presents a lethal mutation with ommatidia that are missing some PRs when examined with rh1-GFP. In addition, the mutant ommatidia often have two R4-like cells (see yellow arrow in Fig. 7D), a defect similar to that observed with Notch driven by the sev enhancer (see Fig. 5F). This cell-autonomous phenotype could be due to a mutation occurring in a member of the Wg pathway that impairs the specification of R3 at the expense of R4.

Fig. 7E presents a mutant baptized fat rhabdomeres (far). It has rhabdomeres that often appear at least twice the size of wild type, and this defect is purely cell-autonomous: we were able to observe mosaic ommatidia with mixed wild-type and disrupted rhabdomeres (not shown). Staining performed in third instar larvae eye disc revealed a wild-type aspect of the developing mutant ommatidia. This mutant therefore most likely affects late steps of PR development, in particular building of the rhabdomere.

Mutants affecting ommatidia polarity
39 mutants were isolated based on defects in ommatidium polarity. The number is fairly small, suggesting that this process involves genes that are required early in eye development. Fig. 7F presents an example of non-cell autonomous effect of the mutant (M80#39, on 3L) tissue on ommatidium polarity. Small clones are seen and often present incomplete ommatidia (missing a few or all PRs). These clones induce a repolarization of the surrounding wild-type ommatidia so that local ectopic equators are generated. As a consequence, ommatidia are found ‘facing’ each other in wild-type tissue, a situation that never occurs in a wild-type eye. This phenotype is comparable with the one presented in Fig. 5C for Iro-C mosaic, although this EMS mutation does not map near the Iro-C locus.

**DISCUSSION**

**Imaging the Drosophila eye**
We report a powerful assay to test the integrity of the ommatidia in living insects with a relatively simple approach. This method has previously been used to study the spectral properties of pigments in bigger flies (Franceschini and Kirschfeld, 1971a; Franceschini and Kirschfeld, 1971b; Franceschini et al., 1981a). We have revisited this technology and adapted it to a large scale for a qualitative and quantitative analysis of genes affecting the Drosophila compound eye. We have shown that the use of auto-fluorescence of opsins and accessory pigments, as well as fluorescence from artificial eGFP transgenes, is a reliable and straightforward method to visualize the ommatidia in vivo. We are able to assay not only the photopigment content, but also planar polarity, proper cell differentiation, morphology and cell survival. This assay is a rapid and non-invasive eye imaging procedure, and requires only a standard epifluorescent microscope.

To illustrate the usefulness of this approach, we have used known examples of abnormal eye organization, in particular, mutations that affect planar polarity or ommatidia morphology. iro-C mutant clones or other genetic backgrounds leading to the impairment of R3-R4 specification (i.e. sev-Nbed or sev-fz flies) adequately illustrate the power of this method. Furthermore, the absence of Rh1 or of inner rhodopsins can also be detected. This point is important, as it allows the investigation of genes involved in terminal differentiation of the different types of PRs (see below).

**Isolation of mutants in an F1 recessive mosaic screen**
We have combined these imaging assays with the powerful tools of Drosophila genetics to set up an F1 recessive mosaic genetic screen to identify mutants affecting PR differentiation or survival (Fig. 7C), PR morphology (Fig. 7D, E), and planar polarity (Fig. 7F) in the eyes of living flies. While it has been shown that genes in the Wg and Notch pathways are involved in the establishment of planar polarity of the eye, the nature of the patterning molecule(s) and many specific downstream components of these two pathways remain to be discovered. Of particular interest is the possibility of isolating homozygous lethal mutation, which is important for screening of mutations that affect PR differentiation and survival, as such genes are likely to be involved in analogous processes in other neurons in the CNS or PNS. Consistent with this, over 70% of the mutations identified in our screen that affect these parameters are homozygous lethal.

While all outer PRs express rh1 and thus can be considered to perform identical physiological functions (Hardie, 1985; Zuker et al., 1985), their sequential differentiation during the development of the ommatidium is clearly different (for a review see Brennan and Moses, 2000). Moreover, although early specification of PRs by sequential activation of the Ras pathway is well understood, terminal differentiation of the different types of PRs still remains obscure. For example, it is important to decipher the regulation of rh1 expression, which is expressed in all outer PRs despite their different developmental origins, and is excluded from inner PRs. This suggests that factors are specifically expressed in outer PRs for their terminal differentiation. Some of these genes, whose
function is restricted to the eye (e.g. nina genes, reviewed by Montell, 1999), has been identified in previous screens but lethal genes could not be identified (Benzer, 1967; Heisenberg, 1971; Koenig and Merriam, 1977; Pak, 1979). As the outer PR neurons specifically project to the lamina part of the optic lobe, genes involved in this specific projection might also be found. Clearly, our ability to detect Rh1 using its auto-fluorescent properties or eGFP transgenes opens the door to the identification of such factors.

Of particular interest are the two principal subtypes of ommatidia (pale and yellow) that are stochastically distributed over the retina. This represents a paradigm to study mutually exclusive expression of sensory molecules such as opsins in a very suitable model: equivalent R7 cells have to make a choice to express either rh3 or rh4, and subsequently communicate this choice to the underlying coupled R8 PRs (Chou et al., 1996; Chou et al., 1999; Papatsenko et al., 1997). It has been suggested that expression of rh4 in R7 constitutes a default state while 30% of the R7 adopt an acquired state, they express rh3 and repress rh4 (Chou et al., 1999). To address this problem, we used an artificial fluorescent transgene (prh3-Rh3-eGFP) to report the pale subtype of ommatidia (i.e. expressing rh3 in R7). Such a setup has allowed us to isolate different mutations that affect the proportion of pale ommatidia in the fly retina (Fig. 7A,B) and are thus potential regulators of rh3 versus rh4 expression in R7.

In our screen, we used green light illumination to assay the integrity of the eyes prior to screening (Fig. 1C,D). By focusing at the surface of the eye, we were able to accurately detect the presence of clones and the morphology of the facet lenses. We then visualized the outer PRs R1-R6 by focusing below the cornea, using alternated exposures to blue/green and UV light (Figs 2B,C, 6E,F). This allowed us to visualize the PRs but also the clonal boundaries (Figs 6E, 7). We then used blue light illumination to visualize in the same eyes eGFP placed under the control of the rh3 promoter (Figs 6F, 7A,B).

The use of these non-invasive techniques has allowed us to perform these analyses in the F1 generation. These methods require simple equipment and white light illumination of the eye can be performed with a simple lamp placed beside the eye to express either rh3 or rh4, and subsequently communicate this choice to the underlying coupled R8 PRs. This allows us to isolate different mutations that affect the proportion of pale ommatidia in the fly retina (Fig. 7A,B) and are thus potential regulators of rh3 versus rh4 expression in R7.

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The use of these non-invasive techniques has allowed us to perform these analyses in the F1 generation. These methods require simple equipment and white light illumination of the eye can be performed with a simple lamp placed beside the standard microscope. The use of rh promoters-eGFP transgenes added to the autofluorescence of opsins offers a powerful and very sustained detection system. Our complete set of rh-eGFP lines considerably simplifies the qualitative and quantitative analyses performed in the Drosophila eye, which otherwise require embedding and sectioning, i.e. the sacrifice of the animal.

The combination of these detection systems with the ingenious setup originally used for F1 or F2 screens to identify mutants affecting PR axon projection (Newsome et al., 2000; Benlali et al., 2000) represents a powerful tool with which to identify even recessive lethal mutations in an easy, rapid F1 screen, with a direct analysis ‘en masse’ of the direct F1 progeny of mutagenized males. In this type of F1 screen, mutant stocks are only established if the mutation is of interest, providing an extremely important advantage that allows the rapid screening of very large numbers of flies.

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