

# Flipping Coins in the Fly Retina

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Color vision in *Drosophila melanogaster* relies on the presence of two different subtypes of ommatidia: the “green” and “blue.” These two classes are distributed randomly throughout the retina. The decision of a given ommatidium to take on the “green” or “blue” fate seems to be based on a stochastic mechanism. Here we compare the stochastic choice of photoreceptors in the fly retina with other known examples of random choices in both sensory and other systems. © 2005, Elsevier Inc.

## I. Introduction

Development of a multicellular organism depends on the proper generation of different cell types. During their life span, cells constantly have to make decisions. These decisions affect cell survival, the commitment to a specific cell fate, and subsequent differentiation, and are made both non-cell autonomously and cell autonomously. In the first case, extrinsic factors, including instructive signals from other cells or tissues and environmental cues, determine cell fate. In the second case, cells make a decision independently of the environment. These intrinsic decisions can be lineage dependent, implying the retention of a molecular memory, or can rely on a stochastic event. In the latter case, the choice can occur between two or more states, and can be preferentially biased toward one of them.

In the case of the *Drosophila melanogaster* color vision system, each ommatidium has to make a stochastic, biased choice between the “blue”

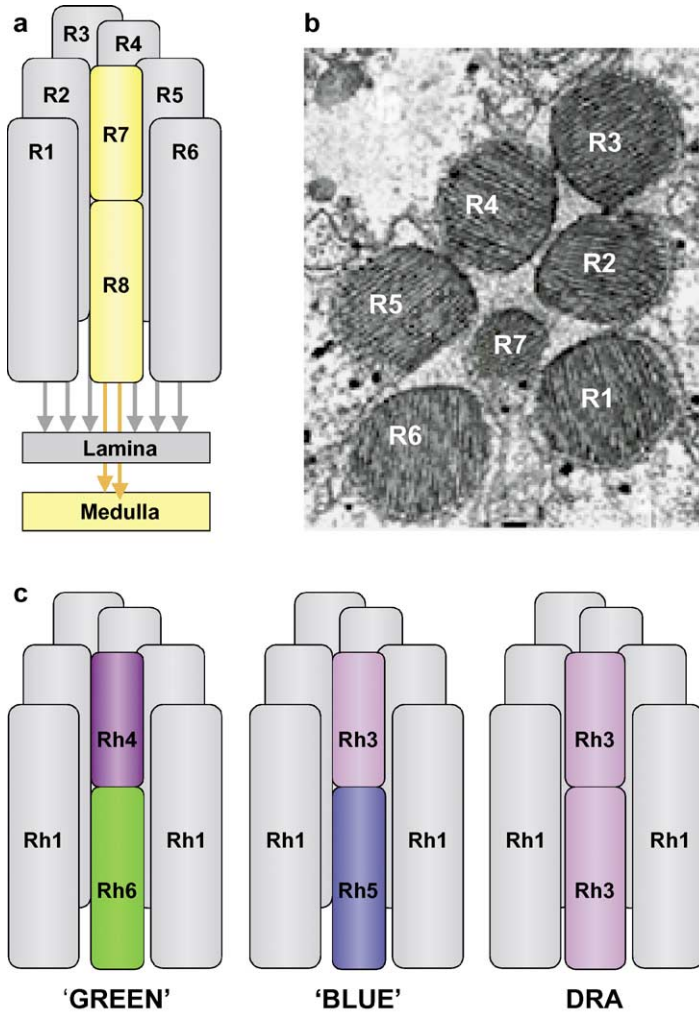
or “green” subtype. This choice does not affect the neighboring ommatidia, which each make their own intrinsic decision. Here we discuss this stochastic choice and compare it to examples of stochastic choice in other systems.

## II. “Green” or “Blue”: A Stochastic Choice in the Fly Retina

*Drosophila* acquires visual information through an array of about 800 ommatidia. Each ommatidium is a single eye unit that has eight photoreceptor cells, a lens, four lens-secreting cone cells, and eight other accessory cells. The eight photoreceptors (R1–8) have widely expanded membranes forming the rhabdomere that harbors the photosensitive G-protein-coupled, seven-transmembrane domain receptor rhodopsins (Rh). Six of the eight photoreceptors (R1 to R6) are involved in motion detection and image formation. The other two photoreceptors, R7 and R8, are involved in color vision and polarized light detection. R1–R6 are called “outer” photoreceptors due to their position within the ommatidium. Their rhabdomeres span the entire thickness of the retina and project their axons to the lamina part of the optic lobe. R1–R6 all express *rh1*, one of the five rhodopsins expressed in the fly eye (Fig. 1a and b) (Hardie, 1985; O’Tousa *et al.*, 1985; Zucker *et al.*, 1985). The morphology and the type of opsin expressed in R1–R6 is invariant in all 800 ommatidia.

R7 and R8 are located in the center of the ommatidium and are therefore called “inner” photoreceptors (Fig. 1a and b). The rhabdomeres of R7 and R8 are much shorter than those of R1–R6, with the photoreceptors projecting to a deeper part of the optic lobe, the medulla. The rhabdomeres of R7 and R8 are positioned on top of each other, R7 being more distal and R8 more proximal (Fig. 1a). An important property of the two inner photoreceptors is that they share a common optic path. When a light beam hits an ommatidium, it first passes through R7 and then R8. It is believed that the fly is able to distinguish colors by comparing the inputs of R7 and R8 coming from one ommatidium (Strausfeld, 1989). R7 and R8 each express only one of four color-sensitive opsins (*rh3*, *rh4*, *rh5*, *rh6*) in a highly regulated manner (Chou *et al.*, 1996; Franceschini *et al.*, 1981; Hardie, 1979, 1985; Papatsenko *et al.*, 1997).

At first glance, the fly retina appears to be a homogeneous structure. However, a close examination reveals that there are three different subtypes of ommatidia (Fig. 1c). The differences are due to rhodopsin expression in the inner photoreceptors (R7 and R8) and their physiological function. Two of the three subtypes, the “green” and the “blue,” are involved in color vision, and their cell fate is chosen by a stochastic event (see below) (Franceschini *et al.*, 1981; Kirschfeld *et al.*, 1978). The third subtype, known as the dorsal rim area (DRA), contributes to the compass of the fly (Labhart



**Figure 1** The three subtypes of ommatidia present in the fly retina. (a): schematic representation of the position, morphology and axonal projection of the outer photoreceptors (R1 to R6) and of the inner photoreceptors (R7 and R8). (b): electron micrograph of a cross-section through an ommatidium. (c): schematic representation of the three ommatidial subtypes present in the retina. In the 'green' subtype (left) R7 expresses *UV-rh4* and R8 *Green-rh6*; in the 'blue' subtype (center) R7 expresses *UV-rh3* and R8 *Blue-rh5*, in the Dorsal Rim Area ommatidia (DRA, right) both R7 and R8 express *rh3*.

and Meyer, 1999). DRA ommatidia differentiation is defined by positional cues rather than by a stochastic event (Wernet *et al.*, 2003). The DRA ommatidia form one or two rows at the most dorsal part of the eye. Inner

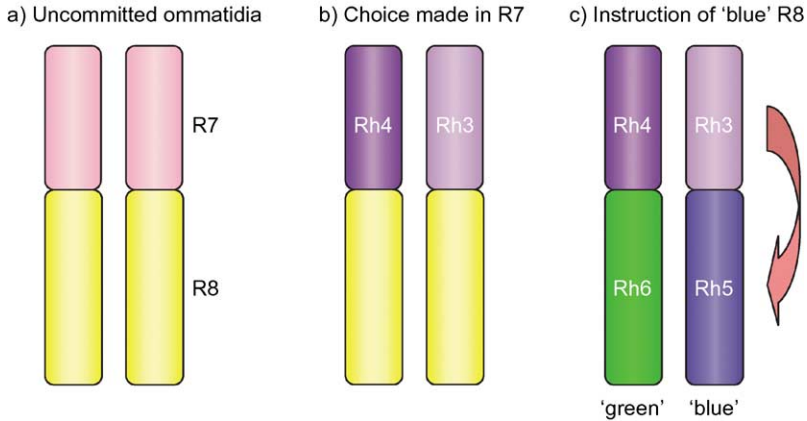
photoreceptors (R7 and R8) of all DRA ommatidia express *rh3* and have a distinct morphology, allowing them to detect the polarization vector of reflected sunlight.

The other two subclasses of inner photoreceptors are involved in color vision. We will refer to them as the blue and the green subtypes; the colors reflect the sensitivity of their respective R8 rhodopsins. Morphologically, the blue and the green subtypes are very similar, the main difference lying in the rhodopsin expression of the inner photoreceptors. In the blue subtype, R7 expresses the UV-sensitive rhodopsin *rh3* and R8 expresses the blue-sensitive *rh5*. In the green subtype, the R7 expresses the UV-sensitive *rh4* (which has a slight shift in the absorbance maximum from *rh3*) and R8 expresses the green-sensitive *rh6*. As in most other sensory systems, each photoreceptor expresses only a single rhodopsin. However, rhodopsin expression within the green and blue subtypes is highly stereotyped. The R7 and R8 rhodopsins are always coupled within one subtype, so that *rh3* is always associated with *rh5* in the blue subtype and *rh4* with *rh6* in the green subtype; however, for example, the combination of *rh4* and *rh5* is never observed in wild-type eyes (Chou *et al.*, 1996, 1999; Papatsenko *et al.*, 1997). Thus, the association of a given R8 rhodopsin with its R7 partner must have a physiological relevance for the fly color vision system. Interestingly, the two subtypes are not represented equally in the retina: 70% of the ommatidia are of the green subtype and 30% are of the blue.

Work over the past few years has elucidated a stepwise genetic model for photoreceptor terminal differentiation. In the first step, the transcription factor *spalt* induces inner photoreceptor (R7 and R8) fate. In the absence of *spalt*, photoreceptors develop into outer photoreceptors (R1–R6) (Mollereau *et al.*, 2001). Then, the transcription factor *prospero* defines the R7 fate by preventing R8 opsins from being expressed in R7 (Cook *et al.*, 2003). After these two steps of cell fate decisions, a photoreceptor knows that it is an inner photoreceptor and that it has become R7 or R8 (Fig. 2a). The ommatidium then has to make one final decision and commit either to the green or to the blue subtype.

Because the two inner photoreceptors of a given ommatidium share one optic path and have to express rhodopsins of the same subtype, the decision must affect both R7 and R8 and must be coordinated between them. Two models can be envisioned: in one, the decision can be made by both cells individually and then coordinated; in the other, the choice is made by one of the cells and is then imposed upon the other one.

The latter appears to be the case. In a *sevenless* mutant in which no R7 cell is present, all R8 cells express *rh6*. In the opposite situation, when R8 is genetically ablated and only R7 develops, both green and blue rhodopsins are expressed in R8. Based on these experiments, the following model was proposed: at the beginning, a stochastic choice between the green and

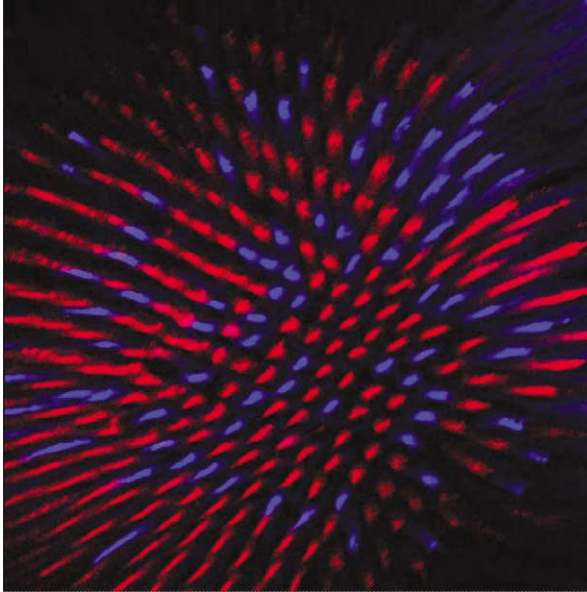


**Figure 2** A two-step model for the cell fate decision ‘green’ versus ‘blue’ in inner photoreceptors. (a): in ommatidia that have not yet committed to the ‘green’ or ‘blue’ fate, R7 and R8 do not express any rhodopsin. (b): a stochastic event in R7 induces expression of either *rh4* or *rh3*. (c): an *rh3* expressing R7 cell sends a signal to the underlying R8 cell inducing *rh5* expression. In the absence of signal (*i.e.* when R7 expresses *rh4*), the R8 cell expresses *rh6*.

blue fate is made by R7 (Chou *et al.*, 1996, 1999; Papatsenko *et al.*, 1997) (Fig. 2b). Once an R7 chooses the blue fate (30% of the cases), it sends an instructive signal to R8. Upon receiving the signal, R8 commits to the same blue fate and expresses *rh5*. In the absence of the R7 signal (*i.e.*, when R7 expresses *rh4*), R8 becomes green (Fig. 2c). This mechanism ensures the correct coupling of rhodopsins between R7 and R8 and does not allow ambiguity. It should be stressed that the stochastic choice is made by each R7 independent of its neighbor, with a bias toward the green subtype, causing it to be chosen twice as frequently as the blue one.

### III. Is the R7 Decision Purely Stochastic?

“Stochastic variation implies randomness as opposed to a fixed rule or relation” (Webster’s Encyclopedic Unabridged Dictionary, 1989, pg. 1398). Is the choice really stochastic? So far, the molecular mechanism of the green/blue choice in R7 has not been elucidated. The distribution of the blue and green ommatidia within the retina allows us to speculate about the nature of the event. The overall distribution of the two subtypes is homogenous over the retina and does not follow any obvious pattern or rule (Fig. 3). No mathematical model has been developed that would predict the fate of a green or blue ommatidium in a specific retinal position, and we can assume that there is no (or only minimal) positional information that influences the R7 decision.



**Figure 3** Stochastic distribution of the ‘green’ and ‘blue’ subtypes in the retina. Confocal image of a wild type whole mount retina stained with anti-Rh5 in blue (‘blue’ subtype) and anti-Rh6 in red (‘green’ subtype). No pattern or rule can be found in the distribution of the two subtypes.

Stochastic choices occur in other circumstances. For instance, in the *Drosophila* nervous system, a single cell is selected randomly from an equivalence group to undergo a specific cell fate. This cell, in turn, prevents the neighboring cells from adopting the same fate through a mechanism known as lateral inhibition (reviewed in [Simpson, 1997](#)). In the fly retina, however, ommatidia of the same subtype can easily be found adjacent to each other, just as ommatidia of one subtype can be completely surrounded by ommatidia of the other. In other words, the fate chosen by a given ommatidium does not prevent adjacent ommatidia from making the same decision, and the ommatidium does not induce its neighbors to make the same choice. This indicates that a mechanism of cell selection followed by lateral inhibition can not apply to the R7 decision: the decision made by a given R7 appears intrinsic, and one can look at each ommatidium as an independent unit.

We assume that the green versus blue choice is based on a stochastic event in R7, with the green subtype accounting for 70% of the ommatidia and the blue for 30%. Therefore, the distribution of ommatidia is stochastic, but is biased toward the green outcome. It is important to stress here that despite the fact that the outcome is binary, the molecular mechanism underlying the choice need not be binary, as more complex scenarios could also lead

to a two-state outcome. A hypothetical example is a stochastic expression of one out of ten transcriptional activators, seven of which would lead to the green fate and three to the blue one. In this hypothetical situation, the event leading to the choice is stochastic and unbiased (0.1 probability for each activator); however, the outcome of the choice is biased toward the green fate.

We can therefore argue that each ommatidium makes an independent decision to become green or blue. The choice seems to rely on a stochastic (random) event. The probability that a given ommatidium becomes green is 0.7 and blue is 0.3. There are other examples in biology where a stochastic choice is made, and knowledge about the underlying biological mechanisms in those examples is useful in helping us understand the development of the fly retina.

#### **IV. How to Choose One out of Two: A Binary Choice in the Primate Retina**

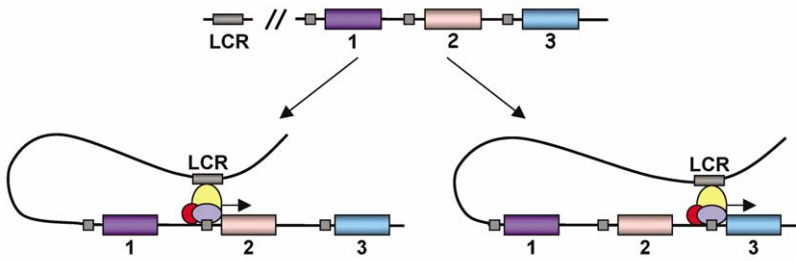
Trichromatic color vision is a recently evolved trait in mammals. In primates, red-green color vision has evolved in two different ways.

New World monkeys possess a single X chromosome-linked green-encoding opsin gene. Within these species, multiple alleles encode different spectral variations of the green opsin. Whereas males possess only one X chromosome and are dichromates, females with a heterozygous set of alleles become trichromates, as different cones express different alleles of the green opsin gene depending on which X chromosome is inactivated ([Jacobs \*et al.\*, 1996](#); [McMahon \*et al.\*, 2004](#); [Smallwood \*et al.\*, 2003](#); [Wang \*et al.\*, 1999](#)).

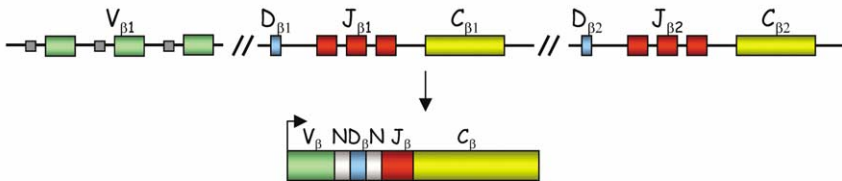
A different mechanism has evolved in Old World monkeys, as well as in humans. In Old World primates, trichromacy relies on the acquisition of a red type (L) of cones in addition to the blue (S) and green (M) cones found in many diurnal mammals.<sup>1</sup> An unequal crossover of two X-linked polymorphic alleles resulted in a head-to-tail arrangement of an M (green) and L (red) pigment gene ([Wang \*et al.\*, 1999](#)). Having the M and L genes on one chromosome requires a mechanism to ensure the expression of one gene in each cone in addition to X-inactivation. The current model for the mutually exclusive expression of the M and L genes involves a shared upstream enhancer termed locus control region (LCR) that escaped duplication ([Nathans \*et al.\*, 1989](#); [Smallwood \*et al.\*, 2002](#); [Wang \*et al.\*, 1999](#)). The LCR regulates the expression of the tandem genes but is able to contact only one of the two promoters through a looping mechanism. The LCR can

<sup>1</sup> In the primate retina, each cone has to make two binary choices: first S versus M/L, then M versus L. The mechanism underlying the first choice is poorly understood and therefore is not discussed here (for further reading see [Bumsted and Hendrickson, 1999](#)).

### a How to choose one from many: the Locus Control Region



### b How to make many from one: DNA Recombination



**Figure 4** How to choose one gene from many and how to make many variants from one gene. (a): schematic drawing of a choice involving a Locus Control Region model. Though a looping mechanism, the LCR can contact only one promoter at a time, inducing the expression of only one gene in a cluster. (b): Schematic drawing of the recombination model in the immune system. The V (Variable), D (Diversity), J (Junction) and C (constant) segments that compose the  $\beta$  chain of the T cell antigen receptor are brought together by DNA rearrangements. Nucleotide addition and deletion (N) in the joining region further increases variability.

function as a stochastic selector for the expression of a single pigment gene from each X chromosome by contacting either the M or the L gene promoter. This allows males to be trichromatic, which is essential for fruit gatherers. Females' X-inactivation is also required so that only one gene is expressed per photoreceptor (Fig. 4a).

The ratio of L and M cones in the human retina is highly variable (Roorda and Williams, 1999). The percentage of L cones is most frequently 65–70% but can range from 50 to 92% (McMahon *et al.*, 2004). How is the different ratio of the two populations generated? McMahon *et al.* (2004) tested the hypothesis that the promoters of the M and L genes carry sequence differences that would allow differential binding to the LCR. Upon closer examination of the 236-bp-long L and M gene promoters from 73 humans, they concluded that sequence polymorphisms could not account for the variability of M and L gene expression. Another hypothesis is that preferential expression of the L gene simply relies on the proximity to the LCR. Although there is huge diversity in the number of L or M genes in the human X-chromosome locus, this is not sufficient to account for the large variability in the ratio (Smallwood *et al.*, 2002), so other factors outside the LCR–L/M

region must also contribute to generate these fluctuations. Interestingly, the extreme differences in the red:green distribution do not affect the color discrimination ability of humans (McMahon *et al.*, 2004; Neitz *et al.*, 2002).

## V. How to Choose One out of Many: Receptor Selection in the Olfactory System

Olfactory receptors (ORs) are, like the opsin proteins, seven transmembrane G-protein-coupled receptors (Buck and Axel, 1991). In flies, about 60 OR genes have been identified, while in vertebrates, the number of identified genes ranges from about 100 in fish to about 1000 in mice and humans. In both cases, the OR genes are distributed throughout the genome, although they are often organized into clusters (Clyne *et al.*, 1999; Gao and Chess, 1999; Glusman *et al.*, 2000; Rouquier *et al.*, 1998; Sullivan *et al.*, 1996).

It is thought that each olfactory neuron expresses only a single OR. Moreover, in mice, only one of the two alleles of each OR gene is expressed in each neuron, a phenomenon known as allelic exclusion, and the choice of which of the two alleles is expressed appears to be random (Chess *et al.*, 1994).

In rodents, the olfactory epithelium can be divided into four zones on the basis of the expression profile of the different ORs: each OR is expressed in only one zone. OR genes can be subdivided into approximately 100 subfamilies, with genes belonging to the same subfamily tending to be clustered together in the genome and expressed in the same zone (Ressler *et al.*, 1993). Within a zone, each gene is then expressed in a certain number of neurons in a stochastic way. When a given neuron expresses one OR gene, it excludes all others, including the other allele of the gene. Therefore, there must be two mechanisms of gene expression regulation: one that ensures that a given OR is expressed in the appropriate zone, and another that is responsible for the stochastic expression of ORs within a zone and for the exclusion of all others.

The presence of *cis*-regulatory elements able to drive expression of a reporter gene in a tissue-specific, zonal, and punctuate fashion similar to the expression pattern of an endogenous OR has been reported for several OR genes (Qasba and Reed, 1998; Serizawa *et al.*, 2003).

Several models that explain expression of a single OR per olfactory neuron have recently been reviewed by Serizawa *et al.* (2004).

The current model for OR selection also involves the presence of an LCR. A 2 kb sequence located 75 kb upstream of the *mOR28* gene (H region) is necessary to induce expression of *mOR28* and of other genes present in the same cluster. This region can activate a single OR gene in the cluster by making contacts with only one promoter at a time, probably through a looping mechanism similar to the one described for the primate retina

(Fig. 4a). The long distance between the LCR and the OR cluster is required to ensure a random selection of all the genes in the cluster instead of a bias toward the most proximal gene. Experimental reduction of the distance between the H region and the OR cluster leads to preferential activation of the most proximal gene in the cluster (Serizawa *et al.*, 2003).

In the mouse and human genomes, there are several OR pseudogenes, some of which can be transcribed. One could imagine that the promoter of a pseudogene instead of a functional OR might trap the LCR, suggesting that until the expression of a functional OR is achieved, the activation process remains active. Upon activation of a functional OR, the process halts. Moreover, expression of a functional receptor initiates a negative feedback loop generating a signal that inhibits expression of the second allele and of OR genes in other clusters (Serizawa *et al.*, 2003).

In other words, in the mouse olfactory epithelium, stochastic expression of an OR gene is achieved through a two-step mechanism: first, a *cis*-regulatory element, the LCR, makes contact and activates only one OR in a cluster; second, the presence of an OR protein somehow inhibits the expression of other OR genes. How this repression is achieved is still under investigation.

## VI. How to Make Many from One: Recombination in the Immune System

Stochastic cell fate choices are not restricted to sensory systems. Another example of large receptor diversity is found in the vertebrate immune system. The mechanism underlying the choice of a single antigen receptor in the B and T lymphocytes is very different from OR selection. While in the olfactory system the choice of one receptor is at the level of gene selection, the diversity of antigen receptors in the immune system is generated by random DNA rearrangement of a single variable coding region.

The T-cell antigen receptor (TCR) rearrangement serves as a powerful illustration of the recombination phenomenon. The variety of the TCR heterodimers (composed of  $\alpha$ - and  $\beta$ -chains) is assembled by somatic recombination from a pool of discontinuous variable (V), joining (J), and diversity (D) gene segments (Fig. 4b). The  $V_{\alpha}$ - $J_{\alpha}$  and  $V_{\beta}$ - $D_{\beta}$ - $J_{\beta}$  rearrangement is based on a stochastic event. The V, D, and J segments are flanked by recombinatorial signal sequences (RSSs), which are recognized by the recombination activating proteins RAG-1 and RAG-2 (Oettinger *et al.*, 1990; Schatz *et al.*, 1989). Further variation is introduced by imprecision in the joining of the coding segments. This junctional diversity is due to nucleotide addition and deletion at the broken DNA ends during recombination. In the case of TCR $\beta$ , allelic exclusion ensures that only a single antigen receptor is

expressed in a given cell (reviewed in [Khor and Sleckman, 2002](#); [Jung and Alt, 2004](#); [Oettinger, 2004](#)).

The theoretical value of combinatorial diversity is calculated to be  $5.2 \times 10^{13}$  possible  $\alpha\beta$ TCR variants in humans. Positive and negative intrathymic selection limit the enormous variability of the T-cells ([Cohn, 2004](#); [Nikolich-Zugich et al., 2004](#)).

To summarize, a stochastic somatic recombination mechanism in the immune system generates a vast diversity of proteins from one single coding region.

## VII. How to Make Many from One: Alternative Splicing of *Dscam*

Another mechanism that produces a large population of different proteins from one coding region is found in the *Drosophila* Down syndrome cell adhesion molecule (DSCAM). Here the variety of proteins is generated from a single coding region by alternative splicing of the mRNA.

*Dscams* are cell-surface proteins containing ten immunoglobulin domains and six fibronectin domains in the extracellular region ([Schmucker et al., 2000](#)). They appear to be involved in axon guidance ([Hummel et al., 2003](#); [Schmucker et al., 2000](#); [Wang et al., 2002](#)). Due to alternative splicing of various exons (e.g., exon 6 has 48 alternative variants; exon 9 has 33) *Dscam* is capable of generating 38,016 possible alternative splice forms, and this diversity is supposed to contribute to the specificity of neuronal connectivity ([Neves et al., 2004](#); [Schmucker et al., 2000](#)). [Neves et al. \(2004\)](#) performed analysis of *Dscam* expression in single cells and homogenous cell populations using quantitative RT-PCR and oligonucleotide microarrays. They found that “a given cell type expresses a broad, yet distinctive, spectrum of splice variants.” As an example, a certain photoreceptor cell may express 14–50 distinct mRNAs from a pool of thousands of exon variants characteristic for its cell type. Thus, the process involves stochastic generation of several splicing isoforms; however, it also implies a random expression of more than one alternative *Dscam* protein from a pool that is specific for the given cell type.

## VIII. Conclusions

Based on the distribution of the green and blue ommatidia, we assume that the R7 choice is a stochastic event, but the exact molecular mechanism underlying the choice is poorly understood. Comparing different systems that base their intrinsic cell decisions on a stochastic event might help us to understand the processes in the fly retina. The examples listed above

elucidate two qualitatively different mechanisms: in primate L/M gene selection and in OR gene selection, the choice is based on selection of one gene among two or among many, respectively. The second case involves the choice from multiple alternatives of a single gene. In the immune system the random selection employs somatic recombination of variable coding segments of a single gene, thus allowing one single choice that is irreversible. In *Dscam* the regulation is posttranscriptional, with alternative exons that are randomly spliced. Moreover, the expression of a subset of *Dscam* splicing variants per cell type, which might also change with developmental timing, adds another level of complexity to the system.

What can we learn from the mechanisms described above? As mentioned before, the blue versus green choice happens in the R7 cell of each ommatidium independently and is then imposed onto the R8, which expresses the matching rhodopsin. In other words, the outcome of the R7 choice is reflected in the expression of *rh3* or *rh4* and leads to the choice of other characters such as the generation of the instructive, blue-specific signal in the *rh3* expressing R7 and the synthesis of a filtering pigment in green R7. One could imagine that the stochastic event selects the blue or green fate at the level of the two rhodopsin genes, as seen in the M/L and OR gene selection. However, the molecular mechanism underlying the phenomenon is clearly distinct: the fly rhodopsin genes do not form clusters and are located on different arms of one chromosome, making the LCR model rather unlikely. On the other hand, no DNA rearrangement nor splicing isoforms have been found in the *rh3* and *rh4* genes. If a similar system were to be used in the fly retina, it would require the regulation of upstream genes rather than that of the rhodopsin genes themselves. In fact, we have recently obtained evidence that a regulator of rhodopsin genes is expressed stochastically in a subset of R7 and precludes the expression of *rh4* (Wernet and Desplan, in preparation). However, the exact biological mechanism for the choice of green versus blue in R7 still remains to be elucidated.

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