Video Article

Preparation of Adult *Drosophila* Eyes for Thin Sectioning and Microscopic Analysis

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Abstract

Drosophila has long been used as model system to study development, mainly due to the ease with which it is genetically tractable. Over the years, a plethora of mutant strains and technical tricks have been developed to allow sophisticated questions to be asked and answered in a reasonable amount of time. Fundamental insight into the interplay of components of all known major signaling pathways has been obtained in forward and reverse genetic *Drosophila* studies. The fly eye has proven to be exceptionally well suited for mutational analysis, since, under laboratory conditions, flies can survive without functional eyes. Furthermore, the surface of the insect eye is composed of some 800 individual unit eyes (facets or ommatidia) that form a regular, smooth surface when looked at under a dissecting microscope. Thus, it is easy to see whether a mutation might affect eye development or growth by externally looking for the loss of the smooth surface ('rough eye' phenotype; Fig. 1) or overall eye size, respectively (for examples of screens based on external eye morphology see e.g.[1]). Subsequent detailed analyses of eye phenotypes require fixation, plastic embedding and thin-sectioning of adult eyes.

The *Drosophila* eye develops from the so-called eye imaginal disc, a bag of epithelial cells that proliferate and differentiate during larval and pupal stages (for review see e.g.[2]). Each ommatidium consists of 20 cells, including eight photoreceptors (PR or R-cells; Fig. 2), four lens-secreting cone cells, pigment cells ('hexagon' around R-cell cluster) and a bristle. The photoreceptors of each ommatidium, most easily identified by their light sensitive organelles, the rhabdomeres, are organized in a trapezoid made up of the six "outer" (R1-6) and two "inner" photoreceptors (R7/8; R8 [Fig. 2] is underneath R7 and thus only seen in sections from deeper areas of the eye). The trapezoid of each facet is precisely aligned with those of its neighbors and the overall anteroposterior and dorsoventral axes of the eye (Fig. 3A). In particular, the ommatidia of the dorsal and ventral (black and red arrows, respectively) halves of the eye are mirror images of each other and correspond to two chiral forms established during planar cell polarity signaling (for review see e.g.[3]).

The method to generate semi-thin eye sections (such as those presented in Fig. 3) described here is slightly modified from the one originally described by Tomlinson and Ready[4]. It allows the morphological analysis of all cells except for the transparent cone cells. In addition, the pigment of R-cells (blue arrowheads in Fig. 2 and 3) can be used as a cell-autonomous marker for the genotype of a R-cell, thus genetic requirements of genes in a subset of R-cells can readily be determined[5,6].

Video Link

The video component of this article can be found at http://www.jove.com/video/2959/

Protocol

1. Fly head dissection

1. Make sure you have all materials at hand (including gelatin coated slides). Prepare glutaraldehyde and Osmium fixatives (see below). Per genotype to embed, aliquot 200μl glutaraldehyde fix solution in 1.5 ml tubes on ice.
2. Anesthetize flies on the CO_2 pad (we usually dissect seven fly heads to embed six per genotype in case one head is destroyed during the procedure).
3. Hold and slightly press the thorax of the fly, dorsal side up, with tweezers and use a sharp scalpel to cut off the head.
4. Stabilize the fly head by touching the neck with the tweezers and carefully slice off a small part of one eye. This enhances penetration of the fixative (if you intend to use your eye sections for clonal analysis, cut the eye with no or smaller clones). Avoid touching and damaging the intact eye that will later be sectioned.
5. Touch the head with tweezers or the scalpel on the surface of the cut off eye and transfer the head into glutaraldehyde/phosphate fixative on ice (the heads often float on top of the fixative) The dissected heads should not be kept in glutaraldehyde fix for longer than 15 minutes prior to the addition of Osmium. Repeat steps 1.2- 1.5 with the other flies of the same genotype.

2. Fixation and embedding (use gloves for all steps!)

1. Spin the flyheads in Eppendorf centrifuge for 1 minute at 10,000 rpm. Continue even if heads don't sink.
2. Add 200µl of OsO_4 solution and fix for at least 30 minutes and up to 1 hour on ice.
3. Using a Pasteur pipette, remove the glutaraldehyde/Osmium solution (make sure to use proper waste disposal procedures!) and replace with 200µl Osmium solution. Incubate on ice for 1-6 hrs. Always ensure the heads are completely covered with liquid and never remove all of the solution as the heads or eyes might collapse!
4. Using a Pasteur pipette, discard fixative, add 0.5-1ml 30% ethanol and incubate for at least 5 minutes on ice (make sure to use proper waste disposal procedures since the ethanol now contains Osmium!).
5. Repeat the above step with 50%, 70%, (80%), 90% and twice 100% ethanol. Include the 80% ethanol step if using the eyes for electron microscopic analysis. After the 70% wash step, the samples can be removed from the ice.
6. Replace the ethanol with an equal volume of propylene oxide (volatile, continue to work in the hood). Incubate for 10 minutes at room temperature.
7. Repeat propylene oxide wash. Work in batches if processing many genotypes in parallel to avoid collapsing of the eyes due to evaporation of the propylene oxide.
8. Remove most of the propylene oxide and using a plastic transfer pipette, add about 500µl of a 1:1 resin:propylene oxide solution. Equilibrate overnight at room temperature.

3. Embedding and arrangement in molds

1. Make sure that all molds are labeled to keep track of your different genotypes, then fill the molds with 100% resin. Do not overfill (no 'high hills' over the surface of the molds when looking straight across the mold surface). Use hard resin for EM analysis.
2. Using a transfer pipette, remove the 50% resin from the heads, replace with 100% resin, and incubate for 3-4 hours at room temperature. As heads are infiltrated with resin, they will sink to the bottom of the tube.
3. Using a toothpick that has been hit on a hard surface once to create a little hook, transfer one head at the time into a mold.
4. Place aluminum foil on the working area of your dissecting scope to protect it against resin spills.
5. Using a dissecting needle and looking though your scope, swirl the head slightly in the resin and carefully move it to the bottom of the mold close to the pointed end.
6. Carefully align the surface of the intact (!) eye (or your future sectioning plane if you want to have cross-sections) neck down with the front wall of the mold, making sure to keep the tangential surface of the eye roughly half a head diameter away from the mold wall. In rare cases, an eye collapses which can be seen as an empty space between the eye surface and the head cuticle. If you have a collapsed eye, replace it with the 7th spare eye.
7. Repeat with all heads. Once done with all alignments, re-check all eyes to make sure they did not move when you removed the needle out of the resin.
8. If you have trouble with eyes moving when you remove the dissecting needle, position the eye as desired, quickly retract the needle slightly away from the head and then slowly remove the needle completely.
9. Bake the molds overnight at 70°C.

4. Trimming and sectioning

1. Remove hardened blocks from the molds by bending the molds, keeping track of which block corresponds to which genotype (e.g. store in small scintillation vials).
2. For trimming, use a chuck appropriate for your microtome that is mounted face up on a block of Plexiglas or aluminum. Mount the block to trim, eye up, in the chuck.
3. Under the dissection scope using a Teflon coated razor bade, carefully cut off only the top layer of your block. This will leave a clear surface though which you should see the eye and can thus predict the future plane of sectioning. Avoid cutting your fingers!
4. Cut off excess plastic on both sides of the head and on the front (i.e. what used to be the top of the mold when aligning the eyes) until most of the plastic around the head is cut off. It is best to slowly approach the head by cutting off multiple thin layers of plastic.
5. Using a clean razor blade, carefully remove thin layers from the top of the eye in the exact plane you want to section later (usually tangentially to the eye, at a slight angle to the original mold surface).
6. Continue until you just start to cut away the outside surface of the eye. Make sure you use clean areas of the blade to obtain a shiny, transparent surface, which makes alignment in the microtome easier.
7. To save on razor blades, use older blades for the crude trimming around the sides of the block and use a fresh blade for the fine trimming (first cut and 4.6). In the end you will have a three sided pyramid with a slightly tilted, cut-off top that corresponds to your future section plane.
8. Mount the block in the microtome. Actual sectioning will vary depending on the type of microtome and will not be described in detail here. We use a Sorvall MT5000 with a histo quality diamond knife to cut 0.5 to 1µm sections. For light microscopic analysis, slight variations in section thickness do not matter. If sectioning clones marked by a White+ transgene, section 1µm sections to ensure to have enough pigment granules adjacent to the rhabdomeres per section (Fig. 2).
9. Sections will float on water. Lift first 20-30 sections with a flattened end of a wooden Q-tip from underneath the water surface in a rotating movement and transfer them into a drop of water on a gelatin coated glass slide (kept on a heating plate at around 100°C).
10. Repeat with another 20-30 sections. Wait until water has evaporated and sections stick to the slide. Usually, sections of 3 eyes arranged in three columns (three eyes) by twp rows (top/bottom sections) fit well on one slide.

5. Staining and microscopic analysis

1. Unless sectioning clones, stain sections. Place slide with sections on a heating block set at 90°C. Dispense Toluidine-blue staining solution from a 30 ml syringe attached to a 0.22µm filter onto the slide and stain for 10 seconds. Immediately rinse extensively with distilled water. Air dry.
2. Using a plastic transfer pipette, distribute 3 drops of DPX mounting medium onto the slide and cover with a coverslip. Top with 3 dimes and let the mounting medium harden (e.g. overnight at room-temperature).
3. Image with a light microscope. Use a 5x or 10x lens to find a good section and then switch to a 63x oil immersion lens for detailed analysis and photographing. We usually use phase contrast settings, but darkfield optics work as well.

6. Representative results:

Most frequently, eyes are embedded in plastic for a detailed analysis of external rough eye phenotypes detected as part of a genetic screen or in the process of testing genetic interactions with genes known to affect either general eye structure or polarity. Typical results of eye sections are shown in Figure 3. Wild-type ommatidia (Fig. 3A) show the full photoreceptor complement surrounded by a lattice of pigment cells. In contrast, in a *strabismus* (*stbm*, a.k.a. van-gogh[7]) mutant, the ommatidial polarity is lost and, even though the full photoreceptor complement is present, rotation and chirality are randomized (Fig. 3B). Furthermore, since the *stbm* allele sectioned was in a w⁻ background, the pigment granules are missing in Fig. 3B. Figure 3C shows an example of a clonal analysis. *Drosophila* Rho kinase (*drok*) is required for ommatidial rotation as well as for the structural integrity of the eye[8]. Homozygous *drok*[2] clones can be identified by the absence of pigment in the pigment cells and the rhabdomeres (typically, clones are induced using *eyeless*-FLP and a P-element with a strongly expressed white marker gene complementing a background w⁻ mutation in the wild-type and heterozygous tissue). It is thus possible to identify mutant areas by their lack of pigment. Due to the cell-autonomy of the pigmentation in the R-cells mentioned earlier, genotypes of individual R-cells can be determined (see arrowheads in Fig. 3C).
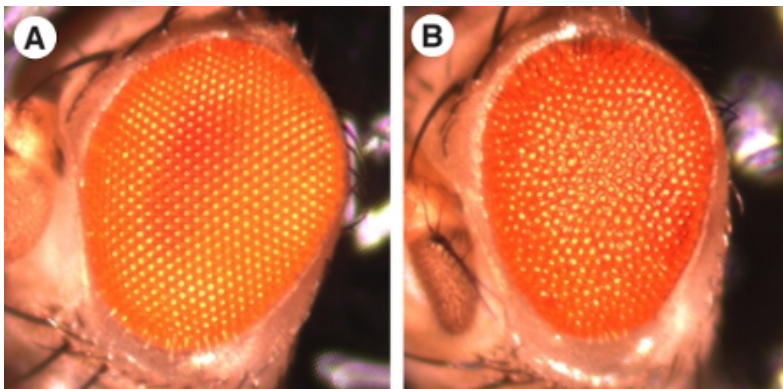

Figure 1. The *Drosophila* eye is an excellent model system for biologists since, in contrast to the smooth surface of a wild-type eye (**A**), the surface of a mutant eye is externally frequently rough (**B**), indicative of underlying eye phenotypes. In all figures, anterior is to the left and dorsal is up. Images Courtesy of Dr. Jennifer Curtiss, NMSU, Las Cruces, NM, USA.
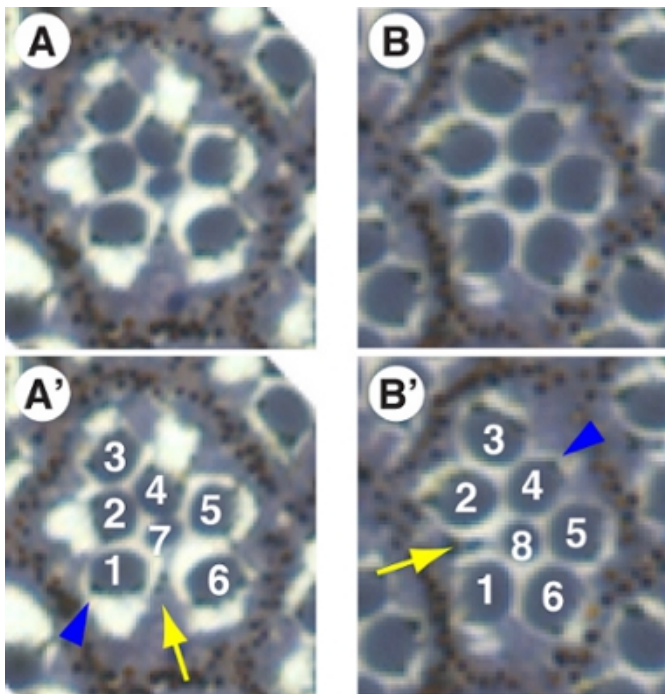
Figure 2. Light microscopic images of single ommatidia sectioned using the described method. Only seven R-cells are visible at a time per ommatidium, since R7 lies on top of R8. (**A, A'**) On the R7 level, the cell body of R7 is detected between R1 and R6 (yellow arrow in A'). In contrast, at the R8 level (**B**), the cell body of R8 is detected between R1 and R2 (yellow arrow in **B'**). Blue arrowheads mark the pigment granules that can be used as a cell-autonomous pigment marker.
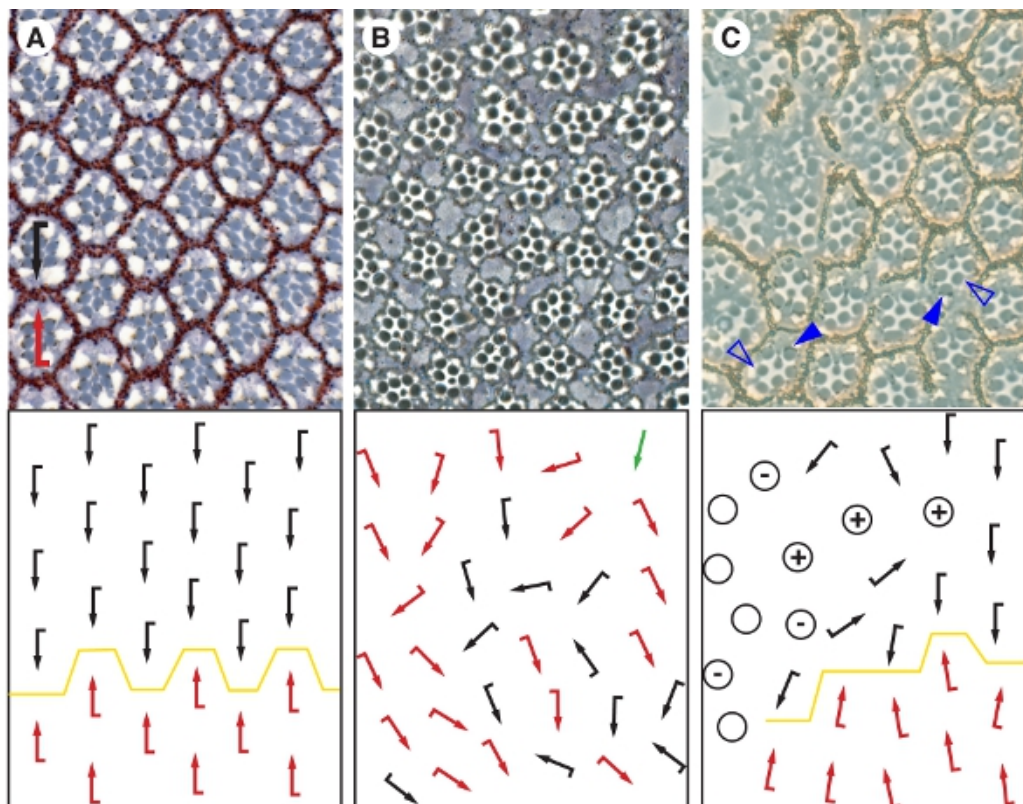

Figure 3. Tangential sections through wild-type (**A**), *stbm* (**B**) and *drok* (**C**) mutant adult *Drosophila eyes*. Schematics below the sections indicate the polarity of ommatidia (see (A) for arrows). Circles represent ommatidia with defects in the photoreceptor complement. Yellow lines represent the dorsal/ventral line of symmetry (equator). In contrast to the well-oriented ommatidia of wild-type (A), the planar organization is lost in the *stbm* mutant (B). Note that the section of the *stbm* mutant shown lacks pigment due to its w⁻ mutant background. (**C**) Because *drok* mutations are lethal, they have to be analyzed in clones. Thus, pigmented cells are wild-type or heterozygous (filled blue arrowheads), while R-cells lacking

pigment (open blue arrowheads) are homozygous mutant. In addition to rotation defects, *drok* mutants also show structural defects including missing or excess numbers of R-cells. Note that for clonal analysis, the sections are not stained to avoid obscuring the pigment granules.

Discussion

Using *Drosophila* as model organism, genetic screens led to the identification of many of the founding members of gene families essential for most of the highly conserved signaling pathways in higher eukaryotes including humans. Since, under laboratory conditions, a functional eye is dispensable for survival, the eye is a particularly well suited tissue for the discovery of novel gene functions and the assessment of genetic networks. Ultrastructural analysis of the fly eye using the described method thus led to fundamental discoveries relevant for development and disease. Initially, single cell clonal analysis was performed using X-ray induced clones combined with known closely associated cell-autonomous recessive markers. More recently, the availability of the FLP/FRT system to generate clones has greatly facilitated the phenotypic analysis of lethal mutations in eye sections[6,9].

Analysis of eye sections is not limited to tangential sections described here. If desired, the heads can be aligned in any orientation in the molds and transverse sections can be obtained to study deeper layers in the head such as the lamina and medulla. The protocol described here is thus a versatile method for analyses of eye and head structures *Drosophila* adults.

Disclosures

No conflicts of interest declared.

Acknowledgements

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