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REGULAR ARTICLE

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A scanning electron-microscopic study of apical contacts in the eye during postembryonic development of *Drosophila melanogaster*

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Abstract The apical surface of the ommatidium plays a major role during development of the compound eye. Cell-cell contacts leading to induction seem to be initiated at this surface. The pupal eye of *Drosophila* was examined, using scanning electron microscopy, from a few hours after eversion of the imaginal disc (19 h after pupariation, 25°C) until shortly after the onset of the corneal secretion (46 h after pupariation, 25°C). At 19 h, the primary pigment cells are in the process of encircling the cone cells. At this time, tufts formed by the cone cell microvilli are the most prominent feature of the eye's surface. Shortly thereafter, the interommatidial cells become more prominent. Their surfaces are raised to form ridges that enclose primary pigment cells and cone cells. From 21 h onwards and lasting for 5–6 h, the interommatidial cells form slim cytoplasmic extensions that spread over the surfaces of the surrounding cells. These extensions contact neighbouring interommatidial or primary pigment cells, but also non-adjacent cells such as cone cells. The fates of these interommatidial cells presumably are determined during that time. The cell-cell interactions may play a role in determining cell fates, for example by providing positional information.

Keywords Visual system · Compound eye · Retina · Eye development · Pupa · Cell-cell contacts · Cell-cell interactions · *Drosophila melanogaster* (Insecta)

Introduction

The compound eye of flies has received considerable attention in recent years. Its highly regular, repetitive structure, the "neurocrystalline lattice" (Ready et al. 1976), has facilitated developmental studies of the component ommatidia at the cellular level. The sequence by which the various retinal cell types differentiate within the ommatidium is well established (for a review, see Wolff and Ready 1993). A growing number of the genes involved in both the determination and the differentiation of the various cell types has for example been identified (for reviews, see Cagan 1993; Zipursky and Rubin 1994; Dominguez and Hafen 1996; Sawamoto and Okano 1996; Freeman 1997). Most attention has focused on the early events that direct development in the photoreceptor cells. Far less is known by contrast about the developmental processes that occur later and lead to differentiation of the cone cells, bristle cell complex and the various pigment cells. Also, little is known about the processes that lead to apoptosis of "surplus" cells that fail to differentiate.

The first ommatidial cells to differentiate are the photoreceptors. Photoreceptor R8 is determined first, followed by the pairs R2/R5, R3/R4, R1/R6. R7 is the last cell to be recruited into the cluster. Once the cluster of eight photoreceptors is established, these cells sink beneath the apical surface of the retina. Anterior and posterior cone cells then form from the undifferentiated cells above each photoreceptor cluster, followed by the equatorial and polar cone cells. Then the two primary pigment cells, recruited from neighbouring undifferentiated cells, become recognizable as they begin to enclose the cone cell quartet. The remaining undifferentiated cells either differentiate into secondary or tertiary pigment cells that separate ommatidia or undergo apoptosis (Cagan and Ready 1989; Wolff and Ready 1993).

There is general agreement that determination of cell fate in the fly's retina occurs through inductive mechanisms, rather than cell lineage (Ready et al. 1976; Lawrence and Green 1979). Determination of the various cells of a bristle cell complex seems to be the only exception to this rule.

Induction has been shown to occur via cell-cell contacts, which seem to be initiated at the apical surface of

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the ommatidial preclusters (Cagan and Ready 1989; Wolff and Ready 1993). The best-studied example is the induction of photoreceptor R7 by R8, via membrane-bound proteins (Reinke and Zipursky 1988; Krämer et al.1991; Cagan et al. 1992). Interaction of the Sevenless receptor in the presumptive R7, with its ligand, Bride of sevenless, in R8, causes the induced cell to differentiate into the R7 photoreceptor.

Signal transduction via diffusible substances also plays a role in cell differentiation (for reviews, see Sawamoto and Okano 1996; Freeman 1997). Spitz and Argos are two secreted proteins, for example, that cause activation, or inhibition of activation, respectively, of the Ras pathway by acting on the *Drosophila* epidermal growth factor receptor. Activation of the Ras pathway via either type of induction is believed to trigger cell-autonomous events such as the activation of transcription factors that cause a cell to differentiate into a specific cell type, R1–7, cone cell, or one of the pigment cell types. At present it is not clear what determines the specification of the particular cell type.

The receptor protein Sevenless is found in the apical membrane of R7, and its ligand, Bride of Sevenless, in the apical membrane of R8 (Banerjee et al. 1987; Tomlinson et al. 1987; Krämer et al. 1991; Cagan et al. 1992). Thus, cell-cell contacts leading to induction presumably occur at this surface. Spitz protein expressed in the developing retinal cells has also been located in the apical tips, as well as in perinuclear granules and the outgrowing basal axons (Tio and Moses 1997). It seems likely that the diffusible part of the Spitz protein involved in induction is released from the apical cell membrane to diffuse in the overlying extracellular matrix.

It seems, therefore, that the apical surface of the eye plays a major role during development. This surface has so far been studied using cobalt sulphide or lead sulphide staining, which outlines cell borders rendering images that resemble line drawings (Cagan and Ready 1989; Wolff and Ready 1991a, 1991b, 1993). These data were used to identify cell types and interpret development. However, while this technique has yielded excellent results, it does not reveal cell surfaces. The few transmission electron micrographs that have been published suffer from the same limitation. Numerous scanning electron micrographs of the adult eye surface and a few scanning images of the surface of a late larval imaginal eye disc have been published (Ready et al. 1976; Banerjee et al. 1987), but a study of the apical surface of the developing pupal eye using scanning electron microscopy has never been reported.

In the present study, I have examined the apical surface of the pupal eye of *Drosophila*, using scanning electron microscopy, from a few hours after eversion of the eye imaginal disc until shortly after the onset of the secretion of the cornea. An interesting stage was observed, when interommatidial cells form numerous apical cytoplasmic extensions that contact neighbouring cells in the ommatidium, as well as cells further away. These extensions may provide the vehicle for cell-cell interactions and thus play a role in determining the fate of the interommatidial cells.

Materials and methods

Drosophila stock

Stocks of wild-type Berlin (*WTB*), wild-type Canton Special (*CS*) and the mutant irreC-roughest^{CT} (rst^{CT}) *Drosophila* were raised at 25°C on blue medium (Boreal) and yeast.

Scanning electron microscopy

Pupae were dissected in cold Ringer's, and brains with attached eye imaginal discs removed. This tissue was briefly washed in a 0.1% protease (Sigma P-5147) in Ringer's solution, rinsed in 0.1 M cacodylate buffer and fixed for 1 h on ice in 2.5% glutaraldehyde, 2.5% formaldehyde in 0.1 M cacodylate buffer, pH 7.3, with 0.02% $CaCl_2$. Brains were washed in 0.1 M cacodylate buffer, postfixed for 2 h in 2% OsO_4 in 0.1 M cacodylate buffer, washed again in 0.1 M cacodylate buffer and dehydrated in a graded ethanol series. Brains were then transferred into a mixture of absolute ethanol/Peldri 1:1 (Peldri II; Ted Pella). After 1 h, brains were transferred to Peldri and the sublimation process allowed to occur overnight. Peldri is a fluorocarbon compound that can be used in lieu of critical-point drying. The tissue was then mounted on carbon tape on stubs, coated with gold/palladium and photographed in a scanning electron microscope (SEM; Nanolab 2000; Bausch and Lomb).

Tissue preparation was somewhat capricious. For example, the removal of the extracellular membrane was not always complete. Also, the sublimation process using Peldri seemed to be disturbed by high humidity resulting in crusty-looking tissue in the scanning-electron microscope. Therefore the best preserved tissue was selected to demonstrate the characteristic features of a certain developmental stage, for which micrographs of the mutant irreC-roughest^{CT} *Drosophila* were also used, provided their mutant features were not clearly visible.

Staging of the pupae

To determine the developmental stages, white prepupae were collected (t=0) and maintained at 25°C for varying intervals till dissection. Age was expressed as hours after pupariation (AP).

In addition to the material that had been carefully timed prior to dissection, some material gained during trial runs has also been included in this study. This material was timed originally using only pupal staging criteria (Bainbridge and Bownes 1981). The exact developmental stage of these pupae was determined subsequently using the ommatidial diameter and other morphological characteristics (see Results).

Results

From the various developmental stages that had been carefully timed prior to dissection, it was possible to derive a growth curve of an ommatidium, measuring the ommatidial diameter across the two primary pigment cells (Fig. 1). The curve shows the increase in ommatidial diameter with age. This varies little between flies of the same age and shows a steady increase in diameter during the first half of pupal development. Besides the ommatidial diameter, flies of the same developmental stage are found to share other morphological characteris-

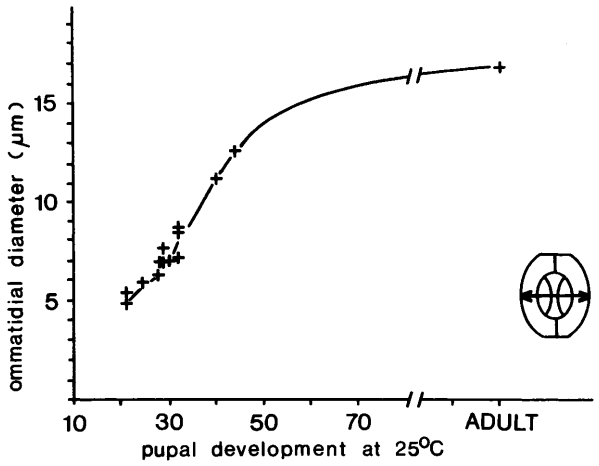


Fig. 1 Increase in ommatidial diameter during pupal development at 25°C. For each data point, the measurements of five different ommatidia were averaged. The diameter was measured across the two primary pigment cells of an ommatidium, as depicted in the *inset*

tics, e.g. a tiny bristle tip is recognizable in all flies studied at 30–31 h AP.

Cells of the younger stages were identified primarily by determining cell identities at 28 h or 32 h AP, when cells are easily identified. From there, they were traced back in time to younger stages where cells are not as readily identified when viewing the stage in isolation, taking into account gradual changes in the appearance of cell surfaces and other developmental changes. Thus the actual research was performed in a sequence reverse from development and its description here.

At the youngest stage studied, 19 h AP, the photoreceptors had already sunk beneath the apical surface and were no longer visible (Fig. 2). The eye's apical surface is formed by the four cone cells and the two primary pigment cells of each ommatidium surrounded by interommatidial cells that are, at this time, presumably still not committed to a specific fate.

Across the eye disc, regularly spaced, whitish tufts appeared prominently (Fig. 2). The tufts are thin, short papillae extending from the surfaces of the four cone cells of an ommatidium. Spaces between the tufts are wide because of the presence of numerous interommatidial cells. The cell surfaces are folded considerably. It is impossible to distinguish individual cells, but cobalt sulphide staining shows that there are at least two or three interommatidial cells separating ommatidia at this stage (Cagan and Ready 1989; Wolff and Ready 1991a). Close to the cone cell tufts, some semilunar-shaped cells, the primary pigment cells, were distinguishable, because their cell surfaces were smoother than those of the interommatidial cells. They were still of relatively small size, enclosing the cone cells only to approximately one-quarter of their circumference, compared with half the circumference encompassed by each of the two primary pigment cells later in an ommatidium. Some cone cell tufts were accompanied by a single primary pigment cell, many by two.

At some time between 20 and 21 h AP, a major change occurred on the apical surface (Figs. 3, 4). At first the image of the apical landscape was still dominated by the cone cell tufts as in the previous stage. Each tuft was now encompassed by two primary pigment cells, with most primary pigment cells enclosing the tufts completely, and only a few not (Fig. 4). Interommatidial spaces werre still very wide, and interommatidial cells still displayed numerous surface folds.

In other pupae of the same developmental stage (21 h AP) though, cone cell tufts had become far less prominent; instead, the topography from this point until their disappearance from view was dominated by the interommatidial cell ridges (Fig. 5). The surfaces of the primary pigment cells (Fig. 6) were largely obscured by the surrounding interommatidial cells, the cell surfaces of which were elevated. These cells started to extend cytoplasmic fingers that contact other cells.

During the next few hours, presumably until approximately 26 h or 27 h AP (Figs. 7, 8, 9, 10, 11), interommatidial cell surfaces formed numerous slim cytoplasmic extensions. These extensions established contact not only with neighbouring interommatidial cells but also with interommatidial cells that were at least one cell removed (Fig. 8). In addition, interommatidial cell extensions spread over the surfaces of primary pigment cells (Figs. 8, 9), where many then dipped into the central ommatidial space, where they contacted cone cells (Fig. 9). Cytoplasmic fingers from interommatidial cells located on opposite sides of an ommatidium also achieved contact above the cone cell tufts (Fig. 10) and in a few cases cytoplasmic fingers were observed actually to extend all the way across the ommatidium to its opposite side (Fig. 11). Occasionally, cytoplasmic extensions from interommatidial cells or a microvillus from the cone cell tufts dipped into "holes" formed by a primary pigment cell because of its irregular shape at these early stages.

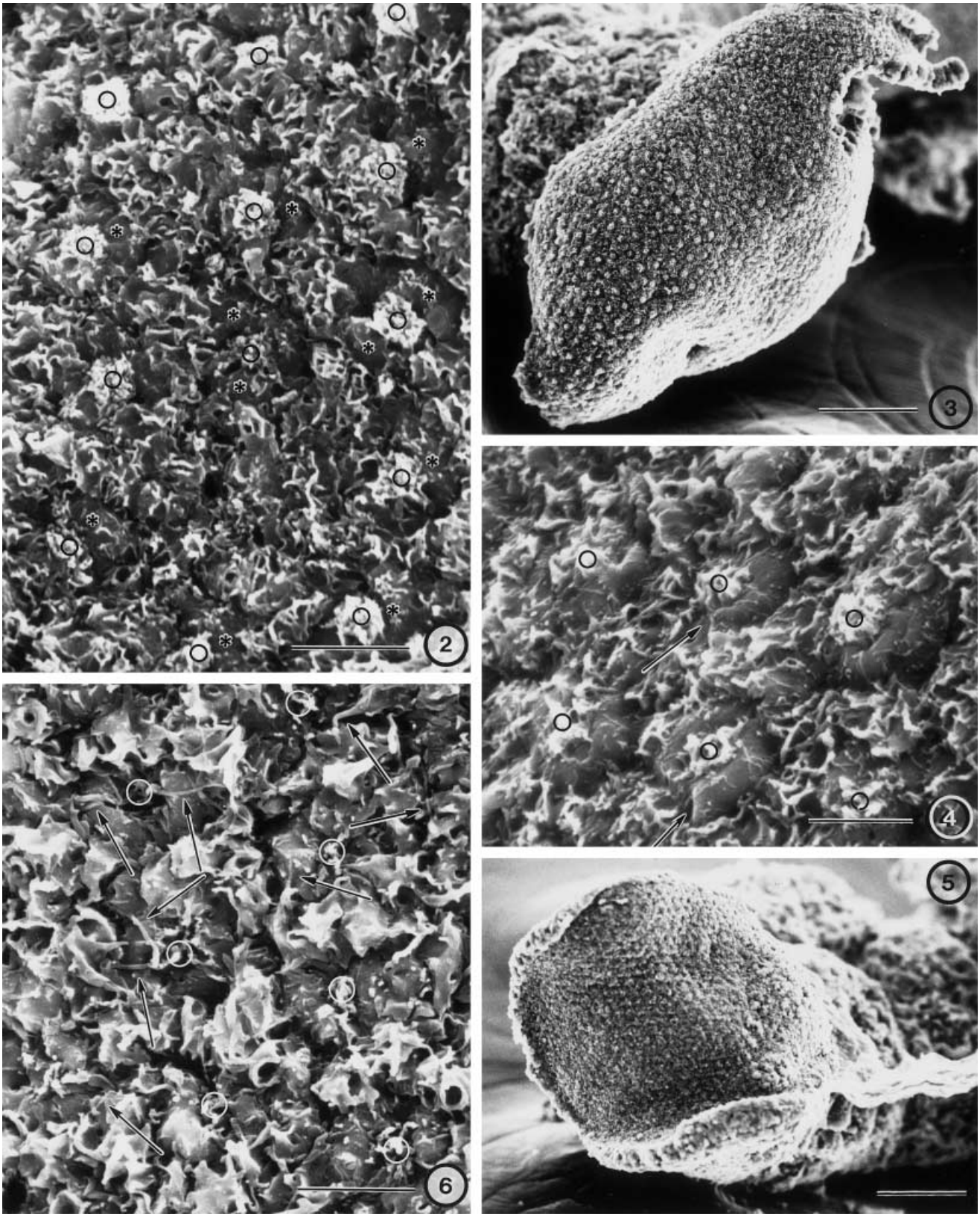
Fig. 2 Apical surface of pupal eye at 19 h AP. Whitish tufts of cone cells (*circles*) are partly enclosed by primary pigment cells (*asterisks*). These clusters of ommatidial cells are regularly spaced within a mass of interommatidial cells. *WTB*. ×4,250. *Bar* 5 μm ▶

Fig. 3 Pupal eye 21 h AP. In this eye, the whitish cone cell tufts are still the dominant feature. *WTB*. ×360. *Bar* 50 μm

Fig. 4 The same eye as in Fig. 3, at 21 h AP, at higher magnification. Regularly spaced cone cell tufts (*circles*) are recognizable. Not all primary pigment cells enclose the tufts completely (*arrows*). *WTB*. ×3,700. *Bar* 5 μm

Fig. 5 Eye of similar age to that in Fig. 4, at 21 h AP, in which ridges formed by interommatidial cells are prominent than the cone cell tufts. *Lower right*, extracellular matrix that covered the surface of the eye remained attached to the edge of the eye. rst^{CT}. ×320. *Bar* 50 μm

Fig. 6 The same eye as in Fig. 5, at 21 h AP. The cone cell tufts (*circles*) and the surfaces of the primary pigment cells surrounding them are almost hidden from view by the raised cell surfaces of interommatidial cells. These cells have begun to extend cytoplasmic fingers (*arrows*) that contact other cells. rst^{CT}. ×4,300. *Bar* 5 μm



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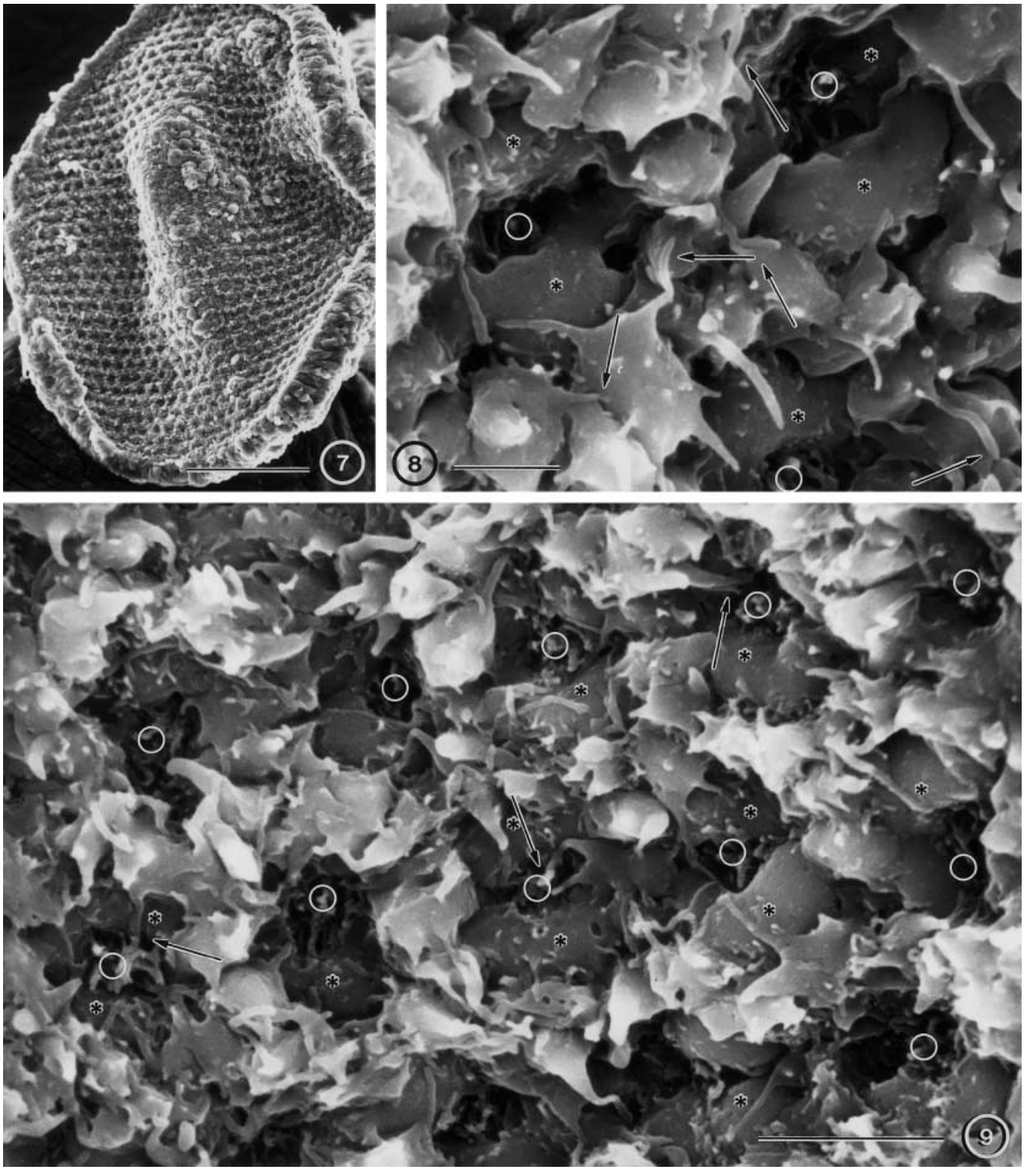


Fig. 7 At about 24 h AP, the ridges of interommatidial cells dominate the appearance of the eye's surface. *WTB*. ×430. *Bar* 50 µm

Fig. 8 Approx. 24 h AP. Interommatidial cell surfaces form numerous slim cytoplasmic extensions. They establish contact with primary pigment cells (*asterisks*), neighbouring interommatidial cells and with interommatidial cells that are at least one cell removed (*arrows*). Some extensions protrude upwards as if reaching into the extracellular matrix. *Circles*, cone cell microvilli. *WTB*. ×9,200. *Bar* 2 µm

Fig. 9 Approx. 24 h AP. Numerous cytoplasmic extensions of interommatidial cells spread over the surfaces of the primary pigment cells (*asterisks*) in the direction of the cone cell microvilli (*circles*), where many establish contact with the microvilli (*arrows*). *WTB*. ×6,500. *Bar* 5 µm

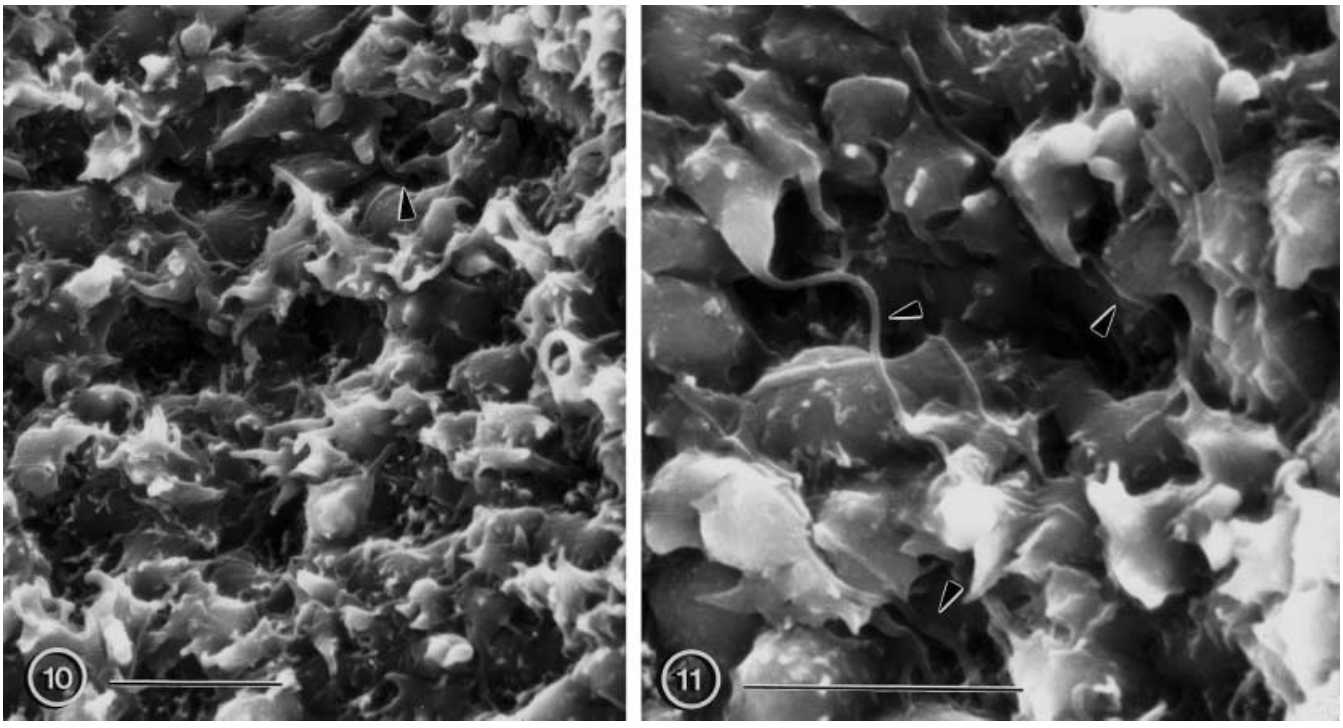


Fig. 10 Approx. 24 h AP. Interommatidial cells from across the ommatidium make contact above the cone cell tufts (*arrowhead*). *WTB*. ×4,500. *Bar* 5 µm

Fig. 11 Approx. 24 h AP. A few interommatidial cytoplasmic extensions cross an ommatidium completely (*arrowheads*). *WTB*. ×7,400. *Bar* 5 µm

A few cytoplasmic fingers were still visible at 27 h AP. Shortly thereafter, by 28–29 h AP, these extensions were gone. The primary pigment cells lost their slightly irregular shape and took on their adult semicircular shape. The ommatidia moved much closer together. The surface area taken up by ommatidial cells increased (see Fig. 1), while the number of interommatidial cells decreased. The interommatidial cell surfaces were still elevated with respect to the surfaces of the primary pigment cells. Many interommatidial cells formed double rows.

By about 28 h AP (Figs. 12, 13), numerous supernumerary interommatidial cells still remained. Whereas previously interommatidial cells formed double rows, most now appeared in single rows, while a few still remained double (Fig. 13). The cells with a small surface (Fig. 13) may have been in the process of dying, because the first signs of cell death are the collapse of the surface, resulting from microtubule loss and a reduction in surface area (Wolff and Ready 1991a, 1993). Note also that some interommatidial cells formed a closer apical contact with their neighbouring primary pigment cells or interommatidial cells than did others (Fig. 13). It was not possible at this stage to distinguish vertices at which bristles would form from those that would contain tertiary pigment cells.

One hour later, at 29 h AP, some supernumerary interommatidial cells still remained. In many flies of this stage, it was possible to determine which vertices would carry bristles and which ones would contain a tertiary pigment cell (Fig. 14). A future bristle vertex was characterized by a gap between interommatidial cells, while alternating vertices contained the future tertiary pigment cells.

At 30–31 h AP, a tiny tip at the bottom of the cavities indicated that the growth of the bristles, i.e. the outgrowth of the trichogen cell, had begun (Fig. 15). An occasional supernumerary interommatidial cell was still seen.

By 32 h AP, the bristle tips (Fig. 16) had grown sufficiently to be easily noticeable, but they did not yet extend beyond the apical surface of the cells surrounding them. Many tormogen cells had encircled their bristle to form the socket. The interommatidial cells looked less prominent, because their apical surfaces were lowered and now lay flush with those of the primary pigment cells. The gap between ommatidia has decreased further and the interommatidial cell surfaces appeared more elongated, rather than square or round. In most flies, their numbers were reduced to their adult complement.

By 35 h AP, the interommatidial cells were still clearly visible. At this stage, and at the subsequent stage studied (40 h), it was difficult to remove the extracellular matrix to obtain a clear view of the surface. Possibly changes had taken place in the matrix related to the secretion of the adult cuticle, as surmised by Locke (1966). Cuticle secretion in most insects seems to start at the tips of microvilli (Locke 1966), or in the case of *Drosophila* at short cytoplasmic protrusions (Bernhard et al. 1970). Waddington and Perry (1960) referred to “pustulate” cell surfaces, which aptly describes the appearance at 35–40 h (Fig. 17). There were numerous cytoplasmic

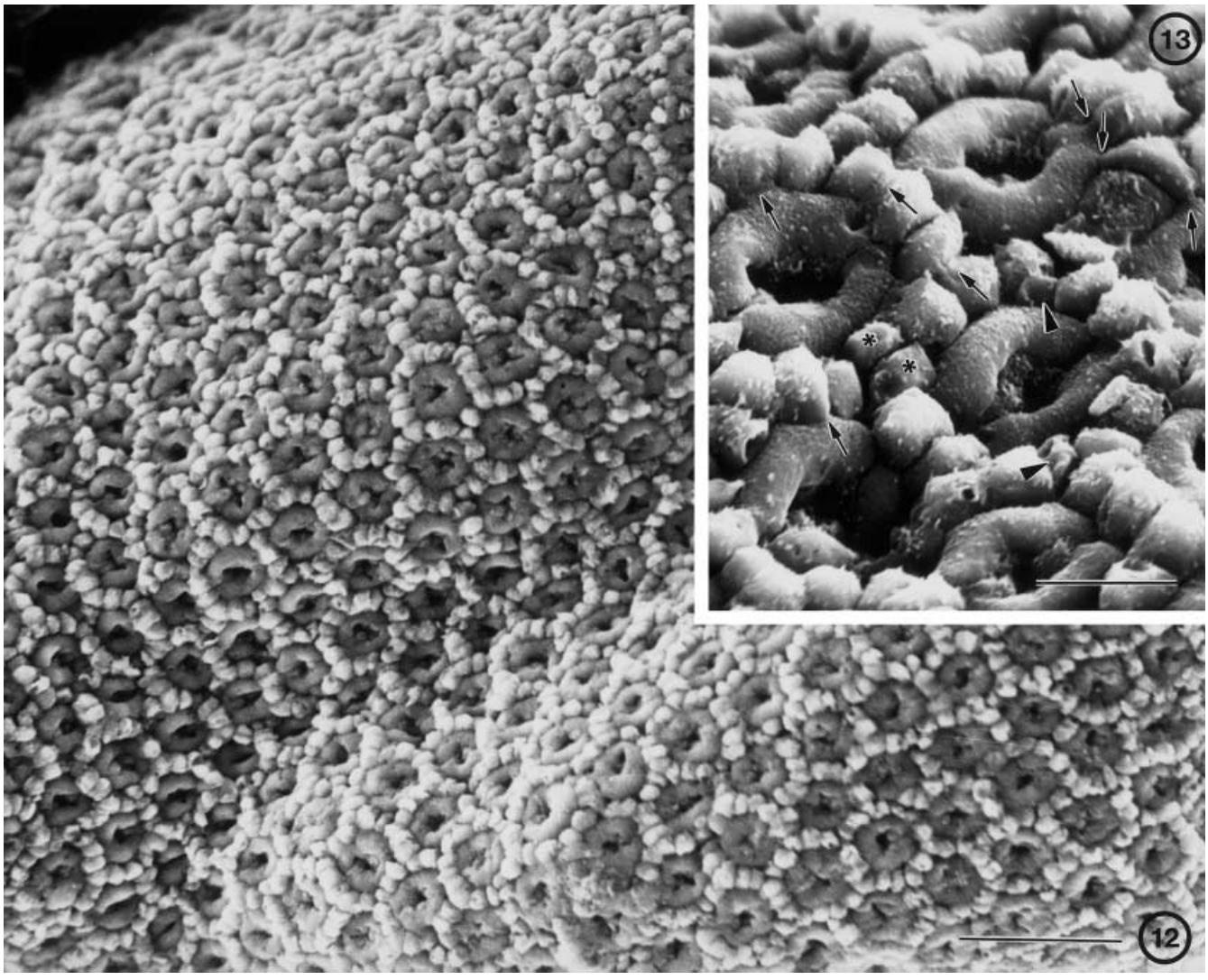


Fig. 12 Approx. 28 h AP. The primary pigment cells have assumed their adult semilunar shape. The interommatidial cells have lost their cytoplasmic extensions. Most now form single rows. They are still more numerous than in the adult. *rst*^{CT}. ×1,200. *Bar* 20 μm

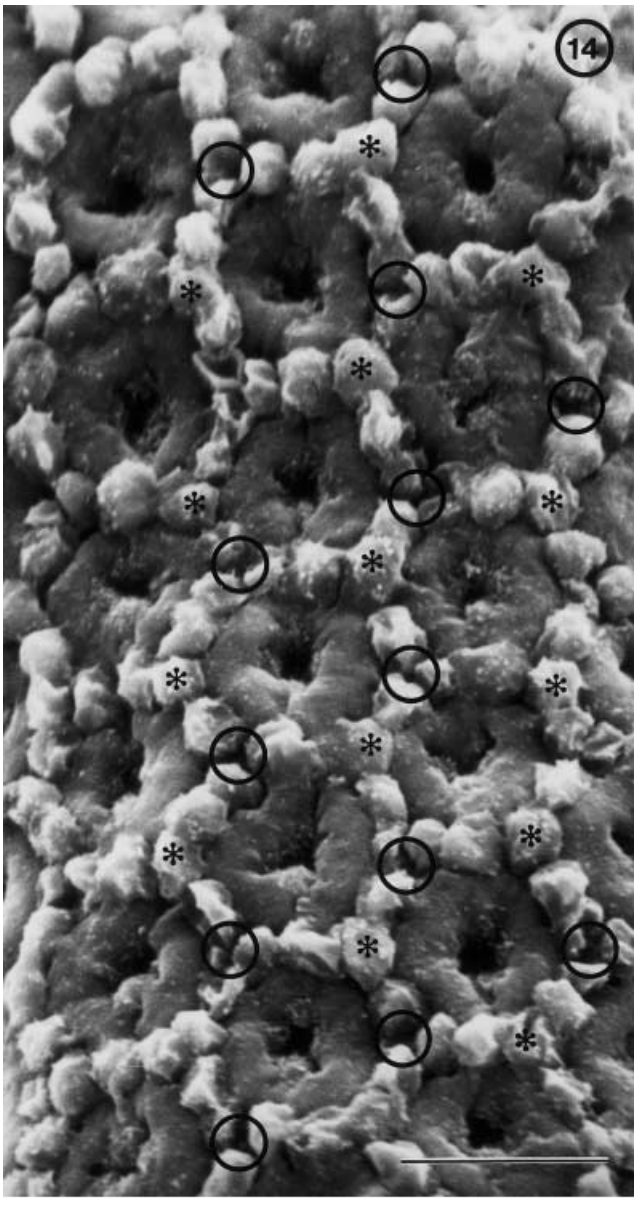
Fig. 13 Approx. 28 h AP. Interommatidial cells have not yet reached adult numbers. A few still lie side by side (*asterisks*); some cells with small apical surfaces (*arrowheads*) are possibly dying. Some interommatidial cells form close contacts with primary pigment cells or other interommatidial cells (*arrows*). *rst*^{CT}. ×4,200. *Bar* 5 μm

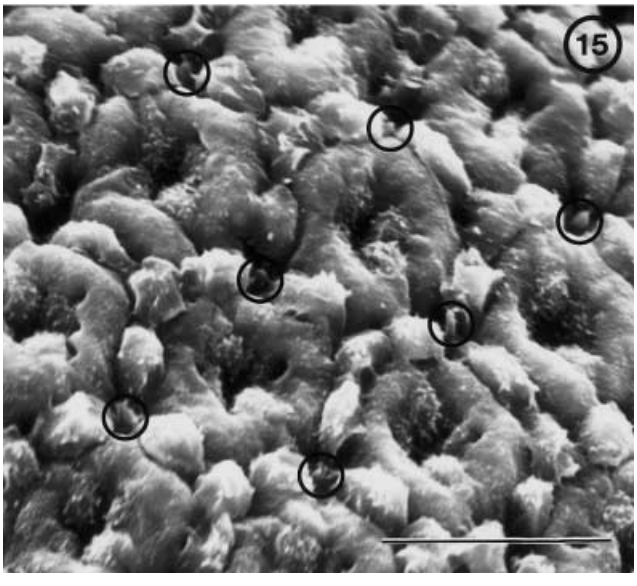
protrusions on all cell surfaces, including secondary and tertiary pigment cells (Fig. 17).

At 40 h AP, gaps between the ommatidia remained; the interommatidial cell surfaces had sunk even lower (Fig. 17). Only occasionally was an interommatidial cell surface visible. The bristles had increased in length. Some of the material which covered the cell surfaces like flaky patches was incompletely digested extracellular matrix. However, most of this material was presumably newly secreted cuticulin, the outermost cuticular layer. Perry (1968a) reports small, dense patches of cuticulin at the microvillar tips of the cone cells at this stage, i.e. the secretion of the adult cuticle has begun.

By 42 h AP (Fig. 18), secretion of the cuticle had progressed to such an extent that a continuous cuticulin layer covered the surface of the eye. Very shallow protrusions about 200 nm in diameter, hexagonally arranged, were recognized on the surfaces. These have been described for the corneal surface in *Drosophila* (Bernhard et al. 1970); in many insects they protrude further and then constitute corneal nipples. Folds of the future cornea were recognizable at the edges of the ommatidia. They increased considerably within the next hour. The gaps between the ommatidia closed and interommatidial cells were no longer detected when scanning the apical surface. The primary pigment cells and cone cells appeared swollen, possibly from material accumulating in the cells prior to secretion or from material already secreted between the corneal surface and the cell surfaces. The bristles appeared longer than at 40 h and had acquired deep ridges (Fig. 19).

At 44 h AP, the secretion of the cornea continued (Fig. 19). It was folded considerably, giving the ommati-





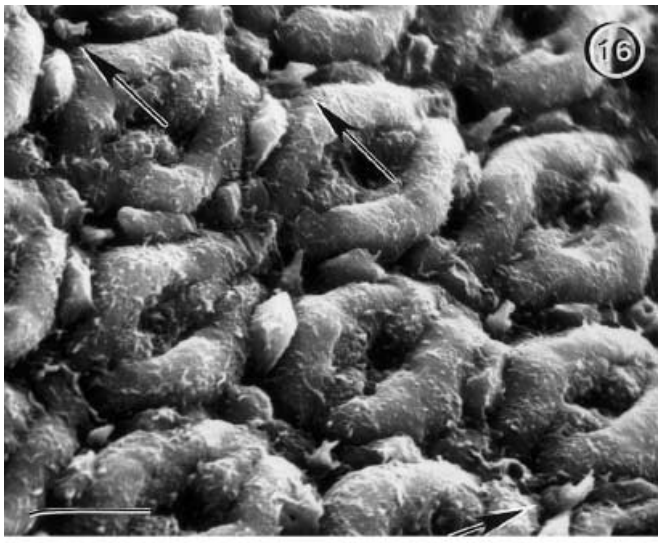


Fig. 16 At 32 h AP. The bristles have elongated and bristle sockets formed (*arrows*). *rst*^{CT}. ×3,300. *Bar* 5 µm

Fig. 17 At 40 h AP. Corneal secretion has begun, visible as flaky patches on the cell surfaces. *WTB*. ×3,100. *Bar* 5 µm

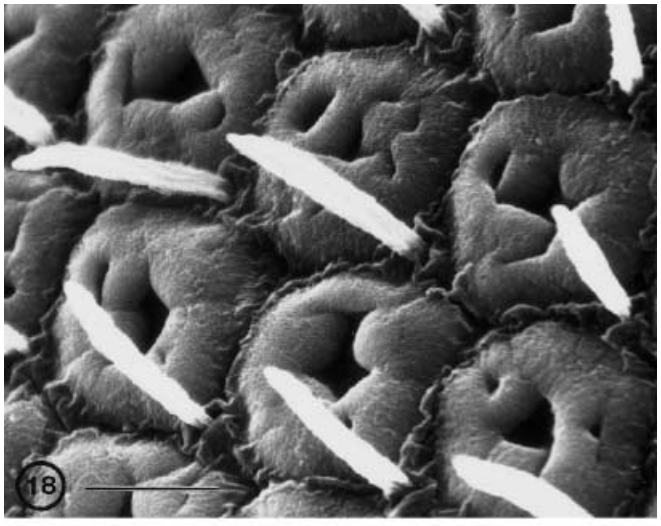
◀ **Fig. 14** Approx. 29 h AP. A few supernumerary interommatidial cells remain. Vertices where bristles will form are characterized by a small gap between interommatidial cells (*circles*), while alternating vertices are occupied by the presumptive tertiary pigment cell (*asterisks*). *CS*. ×2,800. *Bar* 10 µm

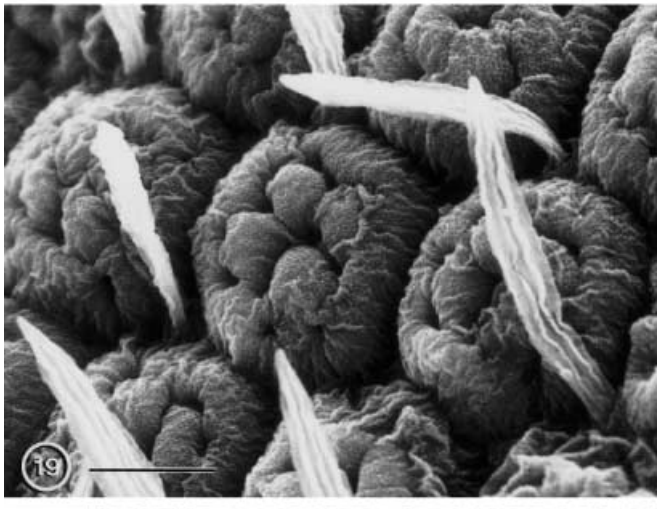
Fig. 15 Approx. 30–31 h AP. Bristle tips appear (*circles*). *CS*. ×3,100. *Bar* 10 µm

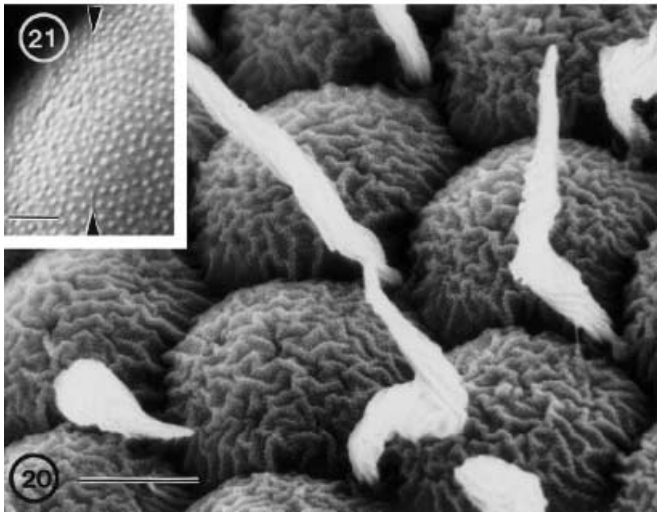
dia a shrivelled appearance. The corneal material laid down, however, was not yet sufficient to obscure the shapes of the underlying cells. Folds form when the surface area of the cornea becomes larger than the surface area of the underlying cells, but is not yet distended by the corneal material that will be secreted between the surface of the cells and the corneal surface (Locke 1966). The bristles increased in size even further.

By 46 h AP (Fig. 20), the oldest pupal stage studied, corneal secretion had proceeded sufficiently to obscure the outlines of the underlying cells. Each ommatidium looked like a wrinkled dome, a shape more reminiscent of its adult form. Cuticle secretion occurs on bristles concurrently with the secretion over ommatidia (Perry 1968b). At this stage, the bristles were already covered by a cuticulin layer, smoothing out the sharp ridges of the bristle to its adult contour.

In the newly emerged adult fly, corneal folds had completely disappeared (Fig. 21). The distance between neighbouring corneal protrusions had increased, suggesting that the surface layer had stretched considerably and possibly that further material was inserted between the protrusions. Irregularities in the spacing of the protrusions gave the impression of fine lines running parallel to one side of the hexagonal base of a facet (Fig. 21). These lines were of unknown origin, but may represent suture lines where corneal surfaces secreted by different retinal cells have fused.







Discussion

This study describes the development of the apical surface of the pupal eye during the first quarter of pupal life. During this time, the eye's surface was not yet concealed by a cuticle, and its cellular landscape was therefore amenable to study by SEM.

Interommatidial cytoplasmic extensions

The most interesting result described here is the finding that interommatidial cells form numerous apical extensions once the two primary pigment cells of an ommatidium have enclosed the cone cells. This occurs at 21 h AP (25°C) and presumably lasts for 5–6 h. The apical extensions grow over the surfaces of primary pigment cells,

Fig. 18 Approx. 42 h AP. A continuous cuticulin layer covers the eye with a granular appearance resulting from shallow protrusions on the corneal surface. The cornea folds at the ommatidial borders. The bristles have increased further in length. rst^{CT}. ×3,800. *Bar* 5 µm

Fig. 19 At 44 h AP. The cornea is considerably folded, with granular corneal protrusions of about 200 nm in diameter. The bristles have acquired deep ridges. rst^{CT}. ×3,900. *Bar* 5 µm

Fig. 20 At 46 h AP. The corneal surface is still folded, but corneal secretion has progressed sufficiently to obscure the outlines of the retinal cells. The bristles have lost their sharp ridges, also due to cuticle secretion. *WTB*. ×3,850. *Bar* 5 µm

Fig. 21 Adult. In the adult the folds have disappeared, and the cornea is stretched out. The distance between the shallow corneal protrusions has increased. Note the difference in appearance between the two *arrowheads*, as if a fine line had been drawn. Possibly these are suture lines, where corneal surfaces secreted by different retinal cells have fused. *WTB*. ×8,200. *Bar* 1 µm

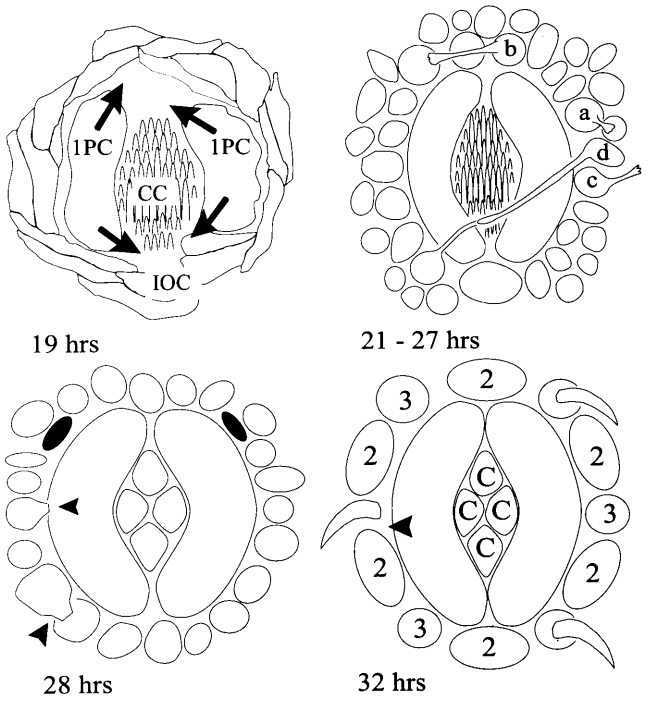


Fig. 22 Summary. At *19 h* AP, the photoreceptors lie beneath the apical surface of the retina. The retinal surface is occupied by cone cells (*CC*), primary pigment cells (*1PC*) and interommatidial cells (*IOC*). At this stage the cone cells of an ommatidium are only recognizable as tufts. Each tuft is partially surrounded by the two primary pigment cells of an ommatidium, which are in the process of enclosing the cone cells (*arrows*). The primary pigment cells are surrounded by interommatidial cells. By *21–27 h* AP, the primary pigment cells completely enclose the cone cell tufts. Individual interommatidial cells are now distinguishable. They display numerous cytoplasmic extensions. Only four are shown here to indicate the types of extensions made: an interommatidial cell contacts a neighbouring one (*a*), or one further away within the same ommatidium (*b*), or one of another ommatidium (*c*). Interommatidial cells also extend cytoplasmic extensions over the surfaces of primary pigment cells, and make contact with the cone cell tufts (*d*). In doing so they may also contact cytoplasmic extensions sent out from across the ommatidium. These contacts between neighbouring and non-neighbouring cells may play a role in determining the fate of the interommatidial cells, i.e. either to undergo cell death or to develop into a secondary or tertiary pigment cell. By *28 h* AP, cone cell tufts are no longer seen; instead cone cells can often be seen as relatively smooth surfaces. The number of interommatidial cells is reduced. Now single rows of interommatidial cells separate the primary pigment cells of neighbouring ommatidia. The long cytoplasmic extensions of the interommatidial cells have disappeared. Short apical contacts are seen between neighbouring cells; two examples are shown here (*arrowheads*). A few cells appear to be in the process of dying (*stippled*). By *32 h* AP, cell death has claimed the last supernumerary interommatidial cells. The ones that remain are located in their adult positions as secondary or tertiary pigment cells (*2, 3*). Bristle tips have appeared, some already surrounded by sockets, others still without (*arrowhead*). By 40 h AP (not shown), only primary pigment cells, cone cells and bristles can still be seen on the surface; secondary and tertiary pigment cells have disappeared beneath the surface. Cuticulin secretion has begun

make contact with cone cell microvilli and even contact distant interommatidial cells (Fig. 22).

Possibly these cell-cell contacts play a role in determining the interommatidial cell fates. Cell-cell interactions are known to influence the differentiation of retinal cells. Also, these apical extensions occur at a time when the fates of the interommatidial cells probably are determined.

Cell-cell interactions

There is considerable evidence that local cell-cell interactions occurring on the surface of epithelia play a major role in cell-fate decisions (Cagan 1993). In the developing eye, changes in contacts between cells are initiated apically (Cagan and Ready 1989; Wolff and Ready 1993), and membrane-bound proteins known to be involved in cell fate decisions in the *Drosophila* eye are localized to the apical surfaces of the cells that express them, including numerous microvilli (Banerjee et al. 1987; Tomlinson et al. 1987; Krämer et al. 1991). Banerjee et al. (1987) speculate that tufts of microvilli might allow developing cells to interact with all the cells in a cluster, not just with their immediate neighbours. Boss protein, located on photoreceptor R8, is internalized by a neighbouring cell, the future R7 photoreceptor, by a process that could involve cytoplasmic extensions, if *boss*-expressing microvilli from R8 were phagocytized by the R7 precursor cell (Zipursky et al. 1992). The non-diffusible form of Spitz also has been located to the apical tips of retinal cells in the developing eye (Tio and Moses 1997). In addition, the diffusible forms of proteins such as Spitz and Argos may be involved in cell-cell interactions (for reviews, see Sawamoto and Okano 1996; Freeman 1997).

Thus, cell-cell interactions of two types, direct cell-cell contact and via diffusible substances, play a role in the development of retinal cells. For both the apical surface seems important.

Timing of cytoplasmic extensions

The cytoplasmic extensions formed by the interommatidial cells occur between approximately 21 and 27 h AP (25°C). At this stage during normal development, there are still three cell fates possible for interommatidial cells: differentiation into either a secondary or tertiary pigment cell, or apoptosis. Several facts suggest that the fate of the interommatidial cells may be determined between 21 and 27 h AP. Heat-shock experiments on *argos* mutants provide evidence that the determination of interommatidial cell fates takes place during the time when cytoplasmic extensions observed in this study occur. Over-expression of *argos* reduces the number of secondary or tertiary pigment cells formed in the eye (Brunner et al. 1994; Freeman 1994; Sawamoto et al. 1994), while loss of *argos* function leads to supernumerary pigment cells (Freeman et al. 1992; Freeman 1994; Sawamoto et

al. 1994). To affect pigment cell numbers, heat shock has to occur between 22 and 32 h AP at 25°C (Freeman 1994), with peak of sensitivity at about 28 h. When the retina was heat-shocked four times at 2-h intervals, starting at 24 h AP, loss of secondary and tertiary pigment cells occurred, while shocks at other developmental stages affected other cell types (Freeman 1996).

These experiments indicate that the differentiation of interommatidial cells into secondary or tertiary pigment cells can be influenced during a time window of about 10 h, from 22 to 32 h at 25°C, during which time the determination of cell fate presumably occurs.

The timing of apoptosis also coincides with the appearance of cytoplasmic extensions. The interommatidial cells are born in the second mitotic wave in the third instar eye disc (Wolff and Ready 1991b), and their death occurs from approximately 35 h to 55 h after pupariation, for flies cultured at 20°C (Wolff and Ready 1991a). It is not possible to convert these data directly to the timing of events at 25°C, because different developmental stages are not all affected in the same way by changing culturing temperature (Bainbridge and Bownes 1981). Nevertheless, the timing of some of the developmental events can be precisely established for both of the culturing temperatures. These include encirclement of cone cells by primary pigment cells and the end of cell death, which proceed between 1.4 and 1.7 times faster at 25°C than at 20°C. The conversion of the commencement of interommatidial cell death at 35 h (20°C; Wolff and Ready 1993) into the corresponding time at 25°C sets the beginning of cell death at 25°C between 21 and 25 h. This is right at the time when apical cytoplasmic extensions are observed. Cell death continues until 55 h at 20°C (Wolff and Ready 1993), corresponding to about 32 h at 25°C, when the numbers of interommatidial cells found here declines to reach the numbers of interommatidial cells found in adult flies.

The relationship between numbers of pigment cells and apoptosis

The decision to undergo apoptosis is linked to the decision not to differentiate into a pigment cell. In eyes with fewer than normal pigment cells due to over-expression of *argos*, increased apoptosis is observed (Freeman 1994), while loss of *argos* expression leads to eyes with increased numbers of pigment cells and fewer cells undergoing apoptosis (Brunner et al. 1994; Sawamoto et al. 1994). A similar effect is seen in *irreC-rst* mutants that have supernumerary pigment cells and decreased cell death (Wolff and Ready 1991a). The time during which increased apical contacts via cytoplasmic extensions were observed in this study (21 h to approx. 27 h at 25°C; Figs. 78, 9, 10, 11) coincides with the period during which these decisions are made. I therefore propose that the decision to differentiate into secondary or tertiary pigment cells, or to undergo apoptosis, occurs with the help of positional information gained by contacts with neighbouring and non-neighbouring cells. During the period from approximately 26 to 32 h after pupariation, when specialized cell contacts can be observed, contacts between immediate neighbours only might be involved in cell fate decisions.

It may be, therefore, that cell fate determination of interommatidial cells is dependent on interactions not only with immediate neighbours but also with cells farther removed, and with cells of at least three different types: other interommatidial cells, primary pigment cells, and cone cells. The role of contacts between non-neighbouring cells has not previously been implicated in cell-cell interactions.

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