F-actin organization during the cellularization of the *Drosophila* embryo as revealed with a confocal laser scanning microscope

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Summary

The changes in F-actin organization during the cellularization of the *Drosophila* embryo have been studied with a confocal laser scanning microscope using fluorescein–phalloidin as a specific stain. Particular study has been made of the changes in the organization of the F-actin network associated with the leading edges of the growing membranes. The role of this actin network in the cellularization process is considered. Other actin-containing structures have also been examined, including the cortical actin layer and a conspicuous region of F-actin aggregates, present beneath the level of the forming cell membranes.

Key words: F-actin, *Drosophila* embryo, confocal microscope.

Introduction

The confocal laser scanning microscope has proved to be of much value in examining the organization of fluorescently labelled cells and tissues (Brakenhoff, 1979; Wilke, 1985; Wijnaendts van Resandt *et al.* 1985; Arndt-Jovin *et al.* 1985; White *et al.* 1987; Jovin and Arndt-Jovin, 1989; Robert-Nicoud *et al.* 1989). This is particularly so with thicker objects for which obtaining images of suitable quality at successive optical planes can be a major problem due to background, out-of-focus fluorescence. With the confocal microscope it is possible to obtain many optical sections of quite thick specimens with little loss of image quality as successive images are built up. This is because of the increase in resolution due to the suppression of the out-of-focus fluorescence with the combination of laser illuminator and detection pinhole. Furthermore, bleaching is minimized because exposure to light is restricted to the area of the preparation being examined. A number of serial optical sections can be obtained and stored digitally in a computer from which three-dimensional reconstructions of specimens can be generated (Brakenhoff *et al.* 1986, 1988; Schormann *et al.* 1989).

Laser scanning microscopy is particularly advantageous for studying eggs and embryos, which, owing to their large size and ultrastructural complexity, are difficult to investigate by conventional techniques. White and colleagues have elegantly demonstrated the value of such an approach in their recent studies of microtubule organization in early stage *Caenorhabditis* embryos (White *et al.* 1987; Hyman and White, 1987). The embryo of the fruit fly, *Drosophila melanogaster* is roughly 450 μm×150 μm in size. In the early stages the embryo is a syncytium. At the beginning of the syncytial blastoderm stage roughly 500 rapidly dividing nuclei migrate into the cortex from the deeper cytoplasm (Zalokar and Erk, 1976; Foe and Alberts, 1983). Each nucleus is surrounded by a cytoplasmic domain, which forms a well-defined 'cap' or 'bud' on the surface (Warn and Magrath, 1982; Foe and Alberts, 1983). A well-developed cytoskeleton is present within each cap, consisting of a thickening of the cortical F-actin meshwork, beneath which is an array of microtubules (Warn *et al.* 1984, 1987; Warn and Warn, 1986; Warn, 1986; Karr and Alberts, 1986; Kellogg *et al.* 1988) and also intermediate filament material (Walter and Alberts, 1984).

After four further divisions the 6000 somatic caps over the whole surface are simultaneously converted into cells by a rather elaborate process. Around each cap the plasmalemma is drawn down into the embryo. All round the leading edges of the caps a triangular vesicular structure, the furrow canal, forms (Fullilove and Jacobson, 1971). The furrow canals surround each cap in a roughly hexagonal fashion and the whole surface becomes packed with hexagonal arrays of inwardly moving membrane with the canal furrows forming the leading edges. Cellularization occurs in two phases, a slow phase during which membrane is pulled down as far as the bases of the nuclei, and a second faster phase that occurs at two to four times the previous rate. The cells that finally form are columnar in shape and linked to the interior of the embryo *via* small intercellular bridges (Rickoll, 1976; Turner and Mahowald, 1976).

The structures involved in the process of cellularization have been studied by a variety of means. A thin layer of

electron-dense filaments has been located very close to the furrow canal membranes using transmission electron microscopy (Fullilove and Jacobson, 1971; Rickoll, 1976). With heavy meromyosin decoration this layer has been shown to contain F-actin microfilaments (Katoh and Ishikawa, 1989). Epifluorescence microscopy after staining embryos with fluorescein–phalloidin has revealed a dense F-actin meshwork around the furrow canals, and also a variety of other actin-rich structures (Warn and Magrath, 1983). However, F-actin-containing structures, particularly those deeper in the embryo, were observed with rather insufficient resolution, due to the very bright out of focus fluorescence, and a number of questions remains concerning these structures. This paper re-examines the changing distribution of F-actin during cellularization as observed with a confocal laser scanning microscope.

Materials and methods

Fixation and staining of embryos

Embryos were collected over 30 min from fly cages and aged for appropriate times between two and two and a half hours. They were then fixed in one of two ways, both methods using 8% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4. Some embryos were punctured in the fixative using a microneedle as described by Warn and Magrath (1983). Others were permeabilized in heptane, fixed with paraformaldehyde and then the vitelline membranes removed with a fine needle after attaching the embryos to the sticky side of electrical insulation tape. They were then stained for 30 min in 5 $\mu g\,ml^{-1}$ fluorescein (FL)–phalloidin (Wulf *et al.* 1979) and mounted in 90% glycerol/10% PBS containing 2% (w/v) *n*-propyl gallate as an anti-bleaching agent. Both fixation methods gave identical patterns of staining.

Laser scanning microscopy

The laser scanning microscope (Carl Zeiss, Oberkochen, FRG) used in this study has been described previously (Robert-Nicoud *et al.* 1989). It is equipped with three laser excitation systems, confocal optics and a high-precision mechanical scanning stage (0.25 μm in the x–y plane and 50 nm in the z axis) operated by an MPC controller through an IEEE interface. The acquisition of data is through an 8-bit, 512/512 frame buffer coupled by an IEEE interface to a central DEC MicroVax II processing system. Image processing was carried out using TCL-image (Multihouse, TSI Amsterdam), an image analysis software developed at the Delft Center for Image Processing. Confocal sections were obtained using an oil-immersion Plan-Apochromat 63/1.4 objective and zoom factors between 1 and 4. Excitation was at 488 nm; fluorescence emission was measured using a FT510 dichroic and a longpass filter (LP 515). Series of up to 40 optical sections, 0.5–2 μm apart, were used to generate stereoscopic reconstructions of the structures observed using the methods of Inoue and Inoue (1986) or Brakenhoff *et al.* (1986).

Results

Fig. 1A–F shows low-power top views and Fig. 2A a high-power side-view of representative FL–phalloidin-stained embryos at the beginning of cellularization. Close to the plasmalemma is an F-actin-rich cortical layer that covers the whole surface of the embryo (Figs 1A, B and 2A, arrow). Because of this layer the apical surfaces of the individual caps are masked. The layer consists of closely packed F-actin material interspersed with more clearly defined small aggregates. It includes the F-actin present

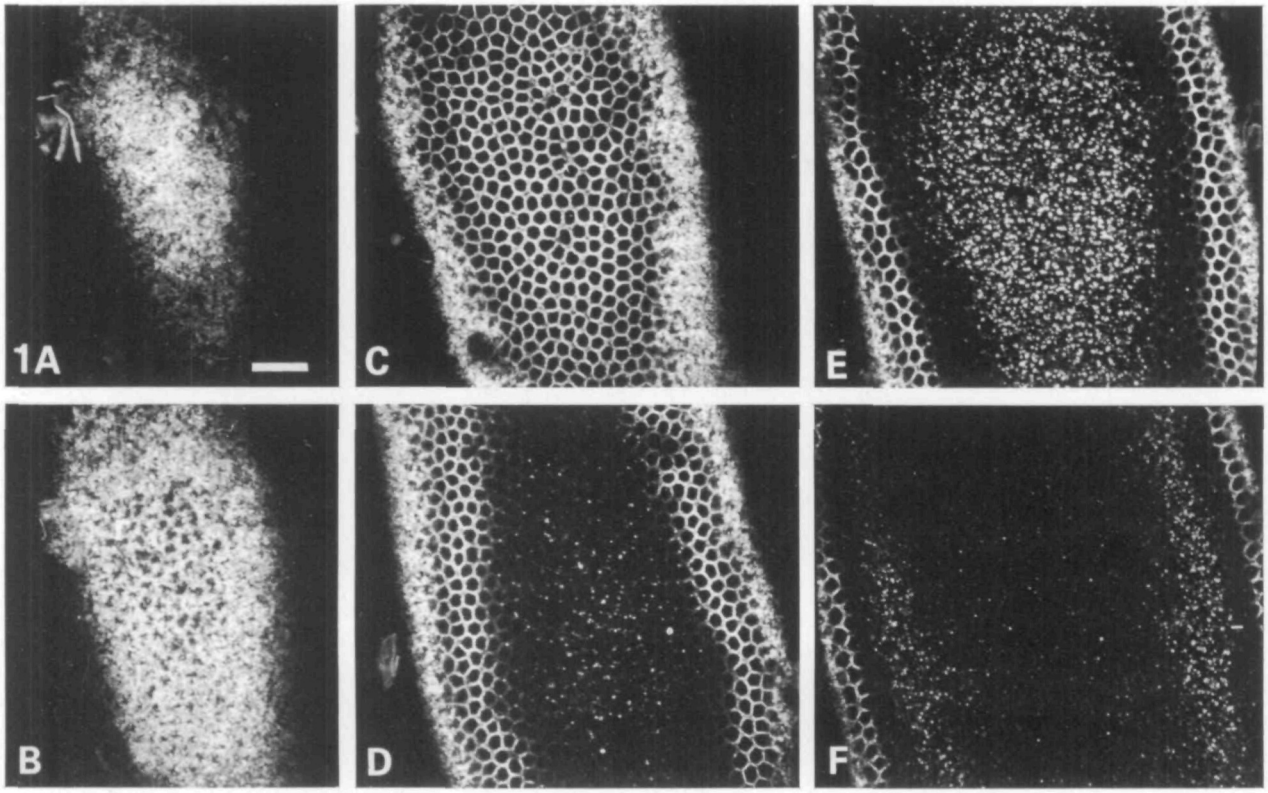

Fig. 1. A–F. Optical sections through a whole mount of an embryo at an early stage of the first phase of cellularization. A. Top surface: B, 2 μm; C, 6 μm; D, 10 μm; E, 14 μm; and F, 20 μm beneath the surface. Bar, 20 μm. (All Figs (except 3) show embryos of various stages of development stained with FL–phalloidin to visualize the F-actin distribution.)

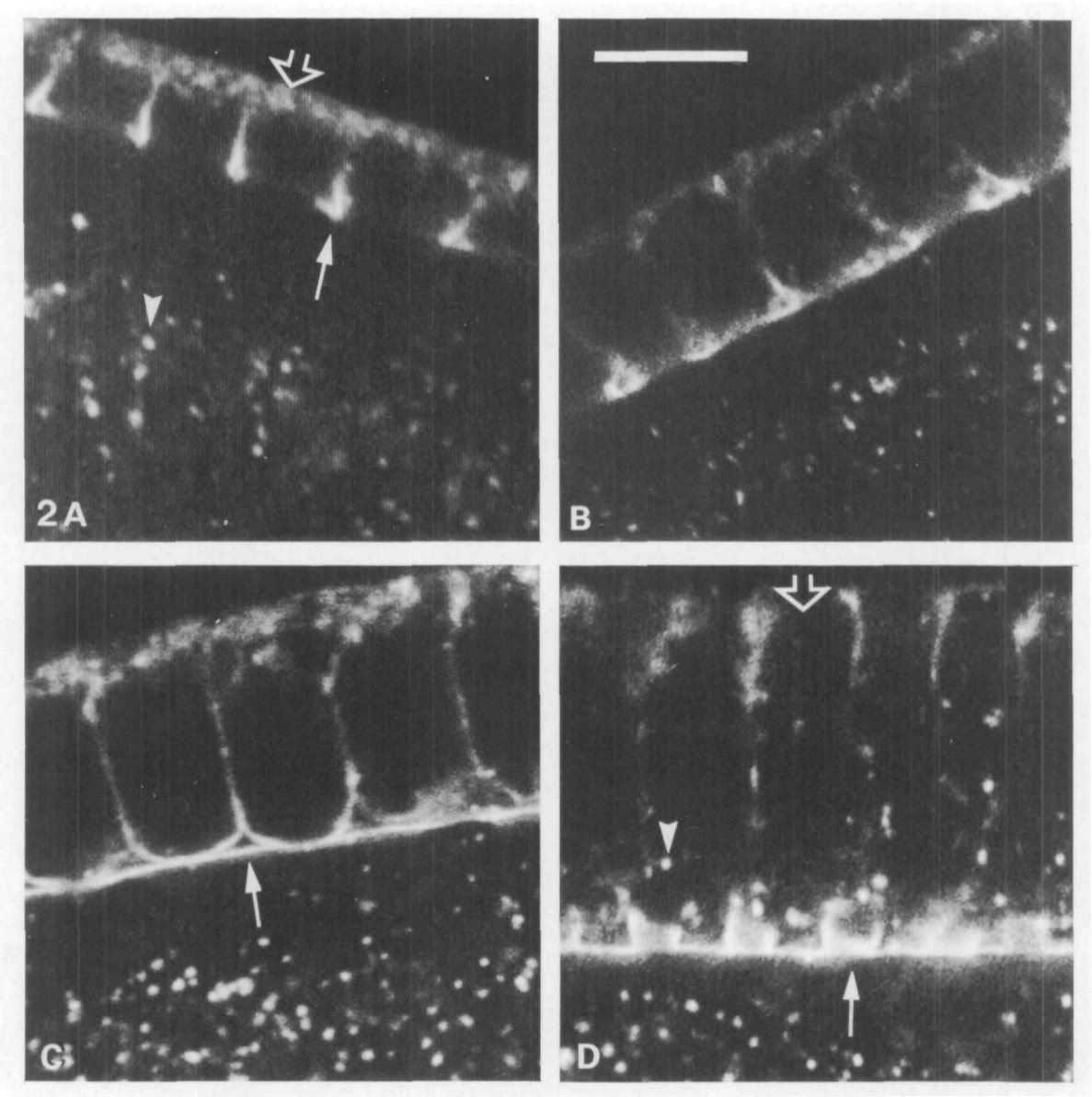

Fig. 2. A–D. Side-views from optical planes at approximately one quarter of the embryo diameter down from the top surface. A. Embryo in the first phase of cellularization. Broad arrow, cortical F-actin; smaller arrow, F-actin around furrow canals; arrowhead, F-actin aggregates deeper within embryo. B. Embryo towards end of the first phase. C. Embryo in the second phase; arrow, expanded furrow canals. D. Newly formed cells; broad arrow, absence of staining associated with apical plasma membrane; smaller arrow, bright phalloidin staining of the side of an intercellular bridge; arrowhead, F-actin aggregates within cell. Bar, 10 μm.

in the cores of the many microvilli that are present at this time as has been previously demonstrated (Warn and Magrath, 1983). The arrangement of the F-actin and its relationship to the plasmalemma at this stage is shown diagrammatically in Fig. 3A.

A second brightly stained layer was found to be present below the level of the cortical layer. It consisted of a network of interlocking rough hexagons or pentagons all over the embryo (Fig. 1C). The position of this layer in the embryo can be assessed from the stereo-pair reconstructions shown in Fig. 4. The layer was visible in side-view as brightly staining irregular triangles (Fig. 2A, small arrow), corresponding to the tips of the ingrowing membranes – the furrow canals (cf. Fig. 3A). In the early stages of cellularization the layer was found to be rather uniformly stained and no sub-structure was visible. Above this layer the newly formed lateral plasmalemmas, which run up to the surface, were also found to be coated with F-actin (Fig. 2A). Below the level of the furrow canals a layer of small F-actin aggregates was present (Figs 1C and 2A, arrowhead). These aggregates were rather variable in size and shape (up to ~3 μm in diameter) and were rather more separated and conspicuous than the F-actin aggregates present in the cortical layer. The aggregates, which were rather more densely packed in some embryos than in others, formed a layer located approximately 5–10 μm below the underside of the furrow canals.

As cellularization proceeds there are changes in the structure of the F-actin network associated with the furrow canals. The inner edges of each hexagon were found to become progressively more nearly circular in form and reduced in size. The inner edges were seen to become

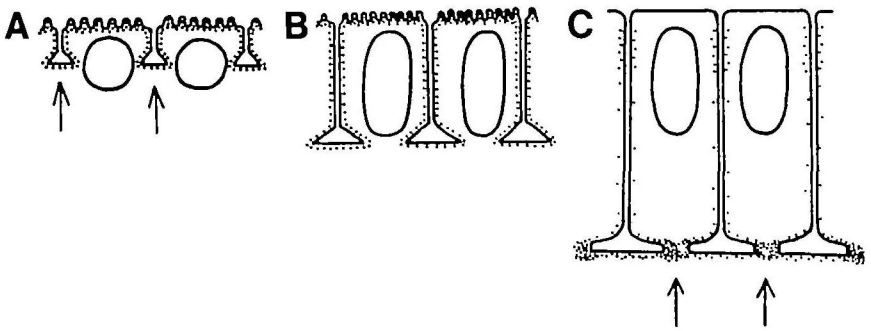

Fig. 3. Schematic representation of the changes in membrane organization during cellularization. A. Early phase I. B. End of phase I. C. Completion of phase II. Arrows in A indicate position of furrow canals. Arrows in C indicate where intercellular bridges have formed. Stippling indicates position of F-actin.

separated by a weakly stained phalloidin layer, which was found to be inserted between the material of the edges (Fig. 5D, arrow). This was due to a gradual enlargement and lateral expansion of the furrow canals and their associated F-actin as best seen in side-view (compare Fig. 2B with A).

Immediately beneath the furrow canals a few F-actin aggregates were present attached to the underside (Fig. 5E). Such aggregates became increasingly numerous further into the embryo as in earlier stages (Fig. 5F). Above the level of the rings the sides of the newly formed plasmalemma were quite strongly stained by FL–phalloidin during this first phase of cellularization (Figs 5C and 3B) and at the apical surfaces the granular F-actin layer remained conspicuous (Figs 5A and B, 2B).

The second phase of cytokinesis starts after the cell membranes have passed the bases of the nuclei. It has been found that the second phase is accompanied by the rapid loss of the layer of F-actin material from the apical surface. Fig. 6A shows a representative embryo at the beginning of the second phase. Less F-actin was seen to be present in the apical actin layer of embryos at this stage than at the maximum development of this layer during the first phase (compare with Fig. 5A). Fig. 6B is of the apical surface of an embryo at the end of the second phase of cellularization where there has been an almost complete loss of F-actin associated with the surface except for a few aggregates and the cell edges are now visible due to actin still being associated with the lateral membrane. The loss of apical actin is also visible in side-view (compare Fig. 2C of an embryo at the start of the second phase and Fig. 2D at the end of cellularization).

The lateral membranes become rapidly lengthened during the second phase of cellularization to a final depth of 30–35 μm within the embryo (Fig. 3C). They can be seen to be lined with F-actin and have rather more actin associated with them towards the apices (Figs 2D and 6C–E). This actin coating was found to be somewhat irregular, with small aggregates being present at intervals along the sides and some thickening at the cell corners (Fig. 6C, arrowhead and arrow, respectively). The lower parts of the nearly complete cells contained small numbers of phalloidin-staining aggregates above the level of the furrow canals (Fig. 2D for side-view and Fig. 6E for top view). Such aggregates were not found within the forming cells prior to the second phase of cellularization.

Further changes were seen to occur at the level of the furrow canals. By the beginning of the second phase the F-actin organization around the furrow canals was much the same as during the first phase, except that the central areas lacking membrane were observed to be smaller and the hexagonal form of the furrow canals had been lost (Fig. 6F). The edges showed stronger staining and were irregularly shaped. As the canals enlarged central circular gaps remained under each forming cell (Fig. 6G). F-actin was found to be deposited on the edges of the widening canals forming rings around the gaps. The gaps mark the forerunners of intercellular bridges. The rings were also visible in side-view as thicker, brighter areas marking the bases of each cell (arrow, Fig. 2D, cf. also Fig. 3C).

By the end of the second phase the canal bases were found to have enlarged further to make fairly large areas of continuous plasmalemma associated with weak phalloidin staining (Fig. 6H). This membrane is known as the yolk membrane and separates the yolk-rich interior of the embryo from the blastoderm (Rickoll, 1976). The ring-

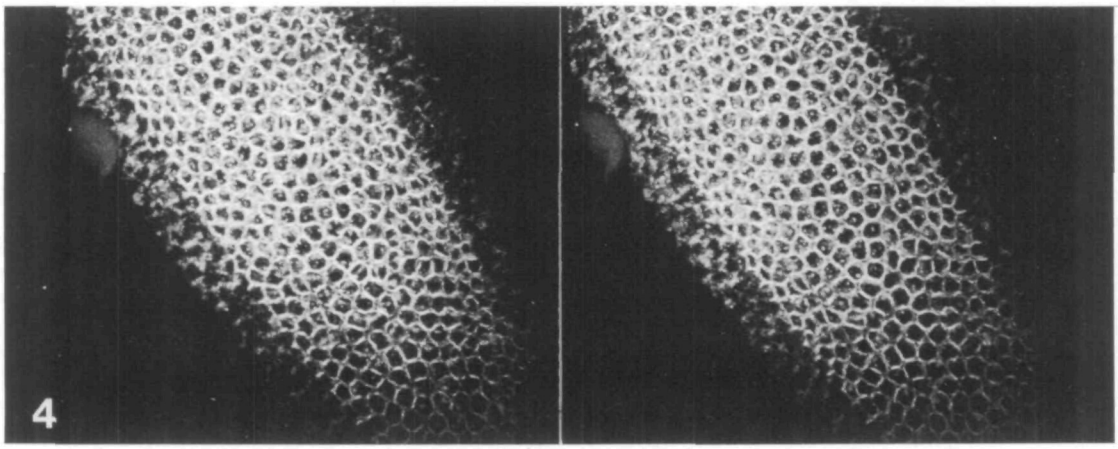

Fig. 4. Stereo-pair of low-power view showing the contractile ring network in relation to the apical F-actin layer above and actin aggregates beneath the network. ×435.

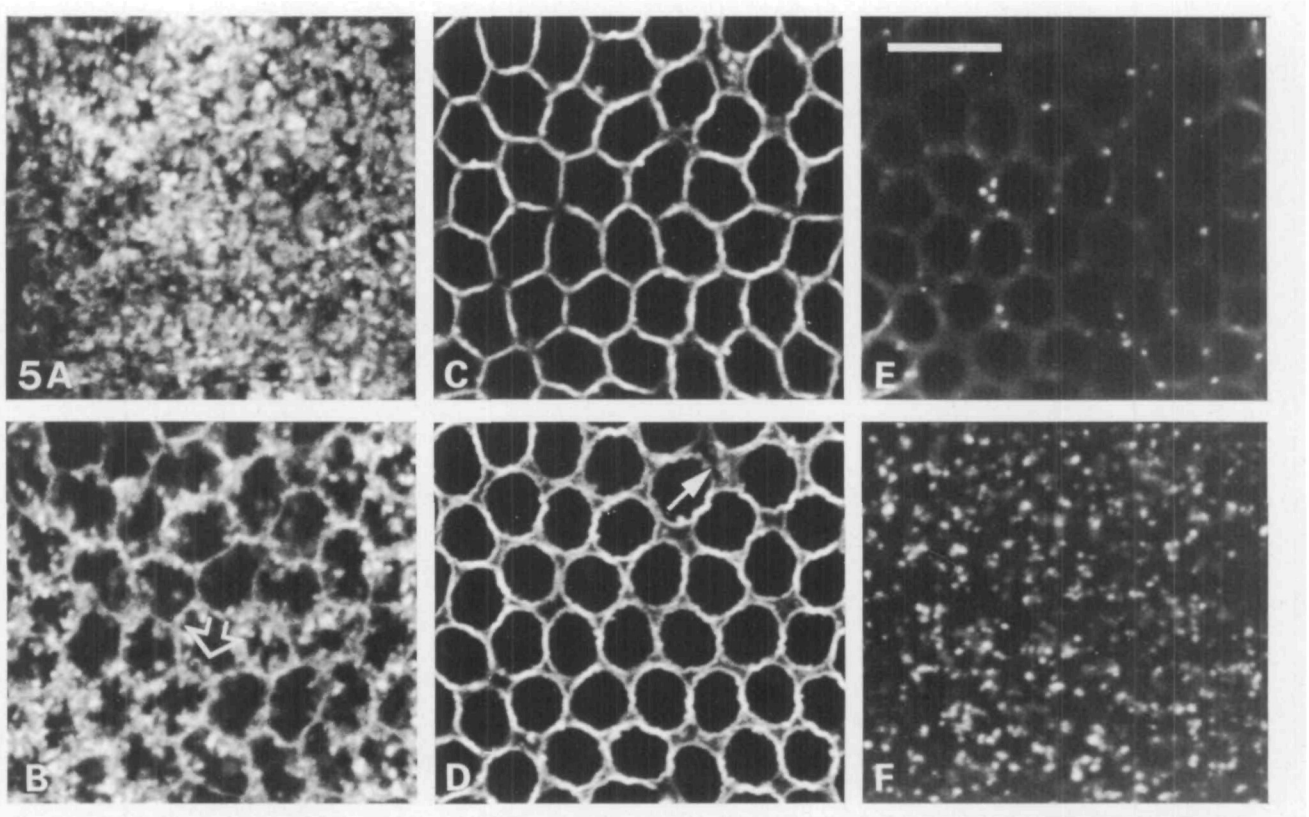

Fig. 5. A–F. Optical sections of an embryo towards the end of the first phase of cellularization. A. Top surface; B, 2 μm below; arrow, granular material in surface layer. C. 4 μm; D, 7 μm; arrow, inserted phalloidin-stained material. E. 9 μm; F, 14 μm. Bar, 10 μm.

shaped actin linings of the central gaps were reduced in size and formed the edges of intercellular bridges (Fig. 6H and I). The bridges were about 1 μm long and the F-actin of their linings stained quite strongly with FL–phalloidin. The bridges link the newly formed cells with the more central regions of the embryos.

Discussion

There is no doubt that the development of the confocal microscope has proved to be a very significant technical step forward, particularly for the examination of larger cells and tissues such as the early *Drosophila* embryo. This can best be judged by comparing micrographs obtained with a conventional epifluorescence attachment with those using a confocal microscope. The use of such a microscope has enabled several features of the changing distribution of F-actin during the cellularization of the *Drosophila* blastoderm (as revealed by FL–phalloidin staining) to be resolved in much better detail. As a result new light has been shed on this rather complex process.

We have reported here that as cellularization proceeds there is a very significant enlargement and lateral widening of the F-actin layer associated with the furrow canals. Eventually the bases of the furrow canals form a large area of plasmalemma, the yolk membrane. The widening of the furrow canals presumably occurs as the result either of the insertion of new plasmalemma and associated cortex material and/or the pulling down of such material into the furrows from above. Fullilove and Jacobson (1971) originally proposed that plasmalemma extension occurs by different mechanisms during the two phases of cellularization. During the first phase the dense layer of microprojections increases significantly at the apical surfaces. It is therefore possible that plasmalemma extension during the first phase may be due to the insertion of new membrane. These authors also showed that the microprojections completely disappeared during the second faster phase and it was concluded that these were pulled out and drawn down to form additional lateral plasmalemma. As shown here and by Warn and Magrath (1983) the loss of surface plasmalemma is also accompanied by the disappearance of the cortical F-actin layer.

Both mechanisms involved in pulling down the membranes could also be involved in the lateral enlargement of the furrow canals. There is also evidence that several organelles may well contribute to the growth of the furrow canals. Coated pits have been found in association with the furrow canals (Fullilove and Jacobson, 1971; Bluemink *et al.* 1985). These are presumed to be derived from the Golgi system *via* transport as coated vesicles and to contribute new membrane material. Multilamellar bodies are also associated with the furrow canals (Sanders, 1975; Bluemink *et al.* 1985) and are thought to fuse with the plasmalemma in the furrow canals *via* focal defects in the lipid bilayer.

It is concluded from this study that two F-actin structures are involved in the formation of blastoderm cells in the *Drosophila* embryo. First, the furrow canals are coated by a dense F-actin layer, which is present around each forming cell and forms a network across the whole embryo surface. This layer also contains myosin (Warn *et al.* 1980; Young *et al.* 1987). Partly because of its composition, and also because cellularization is inhibited by cytochalasin (Zalokar and Erk, 1976; Foe and Alberts, 1983), the

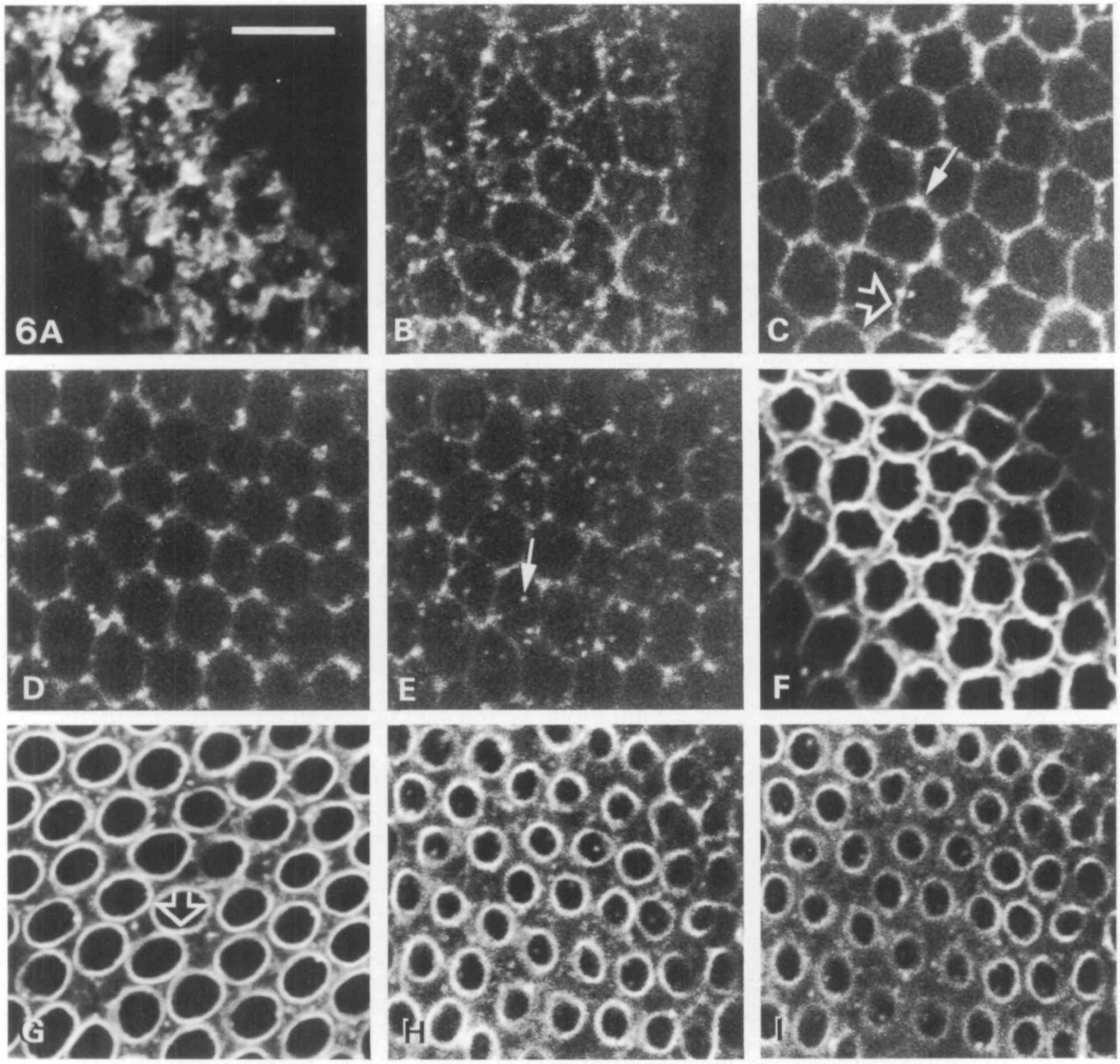

Fig. 6. A–I. Optical sections of three embryos. A,F. Embryo at the beginning of the second phase of cellularization. G. Embryo during the second phase; and B–E,H,I, embryo towards the end of cellularization. A,B. Top surfaces. C–E. Intermediate levels: C, 3.5 μm from top; D, 17.5 μm; E, 21.5 μm. F–I. Level of the bases of the furrow canals; F, 16 μm from top; G, 24 μm; H, 28 μm; I, 29 μm. C. Small arrow, thickening at corners; broad arrow, F-actin aggregates present along sides of cells. E. Arrow, F-actin aggregates within cells. G. Arrow, F-actin lining the side of an intercellular bridge. Bar, 10 μm.

network has been proposed to contract simultaneously all over the embryo as a series of interlocking contractile rings (Fullilove and Jacobson, 1976; Warn and Magrath, 1983). As a result, plasmalemma growth and extension occur behind the furrows, presumably whilst the microfilaments slide past each other within a contractile network (cf. Schroeder, 1975).

As the cell membranes are drawn down the area of the actin network within the embryo becomes reduced. However, this process is accompanied by enlargement of the furrow canals and thus significant reorganization of the network must accompany its contraction. Because of these major structural changes, it is somewhat hard to envisage how the F-actin network could act to pull down the membranes simply by simultaneously contracting all over the surface. A further problem is that micro-injection of anti-myosin antibodies (Lutz and Kiehart, 1987) and rhodamine–phalloidin (Planques *et al.* unpublished) cause localized blockage of cellularization without dramatic effects in other regions of injected embryos. Thus it would seem likely that additional factors, in particular plasmalemma growth and extension, may have major roles in this form of cytokinesis as a 'push' mechanism and that the actin ring network may act mainly to hold the advancing membranes under tension rather than to provide the force for pulling the membranes down. The term 'contractile ring network' has been used elsewhere (Warn *et al.* 1990) to describe this remarkable organization of microfilaments. In spite of the fact that this network seems unlikely to be the sole force required for cellularization, the name

contractile ring network would still seem to be appropriate because of its organization and basic similarities with the contractile rings of other cell types.

During the second phase of cytokinesis it is now clear that a second type of F-actin-rich structure appears. It is formed at the edges of the expanded furrow canals, becomes circular and is then reduced in size. It may well be that these F-actin structures ('rings') are also contractile in nature but act individually rather than in concert. Their contraction would act to pull the base of each cell into a small intercellular bridge. The linings of the intercellular bridges have recently been found to contain myosin (Dr D. Kiehart, personal communication), which is consistent with a contractile role for the rings.

Not only formation of blastoderm cells but also that of the pole cells has been found to occur in two phases (Warn *et al.* 1985*a*). There is first a splitting of the caps, followed by a progressive constriction of the bases, which completely separates off the cells from the embryo surface. Thus, although the mode of formation of the two kinds of cells is rather different, two contractile events would seem to occur in both cases. There are also marked similarities in structure between the cortical F-actin rings of the intercellular bridges attaching the blastoderm cells to the yolk sac and those connecting the nurse cells of the egg chamber to each other and to the oocyte (Warn *et al.* 1985*b*). However, the rings found in the egg chamber expand considerably during the first half of oogenesis, so they are very different entities from a dynamic viewpoint.

The layer of F-actin aggregates that lies beneath the furrow canals and also that associated with the apical cap surfaces have been revealed in much more detail with the confocal microscope. The conspicuous irregular aggregates are very similar to those found around the caps during syncytial blastoderm and also form part of the cortical F-actin layer at the pre-blastoderm stage (Warn *et al.* 1984; Warn, 1986; Karr and Alberts, 1986). Somewhat larger aggregates are also found in late-stage egg chambers (Warn *et al.* 1985*b*). F-actin aggregates are a conspicuous feature of a variety of cell types in a very wide range of organisms. They have been found in the cortex of amphibian oocytes (Franke *et al.* 1976), and early stage mammalian (Lehtonen and Badley, 1980) and nematode (Strome, 1986) embryos. They occur in yeast (Kilmartin and Adams, 1984), fungi (Hoch and Staples, 1983) and also transformed cells (Boschek *et al.* 1981; Carley *et al.* 1981, 1985).

The finding that similar size classes of irregularly shaped F-actin aggregates occur in such a range of organisms implies some kind of common ultrastructure and, perhaps, function. In the *Drosophila* embryo several pieces of evidence suggest that the deeper cytoplasmic aggregates may be a form of actin storage. The layer becomes significantly sparser by the end of cellularization. This suggests that their material becomes included in the layer of F-actin-rich material associated with the furrow canals and with the lateral plasma membranes. Aggregates are found within and above the actin-rich layer of the furrow canals during the second phase of cellularization, seemingly in transit. Micro-injection of phalloidin into embryos undergoing cellularization causes some loss of the aggregates and also the polymerization of additional actin around the furrow canals (Planques *et al.* unpublished). On the basis of similar experiments in which phalloidin was injected into mammalian cultured cells, Wehland and Weber (1981) have surmised that F-actin may exist in short polymers that are transported from one area of a cell to another. Perhaps actin exists in such a form within the aggregates and the short polymers are released during cytokinesis to form part of the F-actin polymers around the furrow canals.

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