Distribution of F-Actin during Cleavage of the *Drosophila* Syncytial Blastoderm

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ABSTRACT   The process of cleavage during the syncytial blastoderm stage of the *Drosophila* embryo was studied in fixed whole-mounts using a triple-staining technique. Plasmalemma was stained with Concanavalin A conjugated to tetramethylrhodamine isothiocyanate, the underlying cortical F-actin with a fluorescein derivative of phalloidin, and nuclei with 4',-6 diamidine-2-phenylindole dihydrochloride. The surface caps, which overlie the superficial nuclei at this stage, were found to be rich in F-actin as compared with the rest of the cortex. After the caps formed, they extended over the surface and flattened. Whilst this was occurring the F-actin network within the caps became more diffuse. By the end of the expansion process F-actin had become concentrated at both poles of the caps. The caps then split in two. The cleavage was not accompanied by the formation of any apparent contractile ring of microfilaments across the cap, rather the break region was depleted in F-actin. The cortical actin associated with each half of the old cap then became reorganized around a nucleus to form a new daughter cap, and the cycle began again.

The development of the *Drosophila* embryo consists of an initial stage when nuclei cleave rapidly within the egg but no cell membranes are formed (6). After the ninth cleavage, ~400 nuclei migrate to the surface. Between 10 and 20 nuclei located at the posterior tip become incorporated into pole cells, which are the progenitors of the germ cell line. The remainder become associated with cytoplasmic caps or protrusions that bulge from the surface (4, 13, 16, 21). These caps are surface structures only, being specializations of the cortex. This stage is known as the syncytial blastoderm. It lasts for ~1 h, during which the nuclei and associated caps undergo four further cleavages. The caps go through cycles of expansion and flattening, taking in the whole of the surface prior to dividing and rounding up again (16). After this stage the whole of the embryo surface becomes cellularized simultaneously.

The syncytial blastoderm has several useful features for structural studies of cleavage and mitosis. Because the caps are surface structures and the nuclei are <5 μm from the plasmalemma it was possible to observe clearly these structures with epifluorescence microscopy. Divisions occur rapidly between 8 and 20 min apart, the earlier ones cleaving more rapidly (16). Furthermore, in a proportion of the embryos cleavage is not synchronous but occurs in gradient-like fashion, with various different stages present in a single embryo (21). As a result it was not difficult to find embryos with caps at all stages of division.

In a previous report (17) the distribution of F-actin microfilaments was described during the process of blastoderm cellularization using a fluorescein derivative of phalloidin (FL-phalloidin)[1] as a specific stain for F-actin. This paper examines the organization of cortical F-actin during the preceding syncytial blastoderm stage.

MATERIALS AND METHODS

Embryos of the desired stage were manually dechorionated and attached to a siliconized slide with glue from PVC tape dissolved in heptane. After ~5 min of desiccation over silica gel at 25°C, the embryos were covered with 8% formaldehyde in PBS. The formaldehyde was prepared from paraformaldehyde and stored until use at −70°C. The embryos were then punctured mid-laterally to allow rapid fixation. This was done with a glass microneedle attached to a de Fonbrune manipulator. The needle had a fine tip and then widened to ~15 μm. Thus a small puncture could be made without noticeable loss of cytoplasm. After a 30-min fixation at 25°C, the vitelline membrane was manually removed and the embryos were thoroughly washed with PBS.

Staining: Embryos were stained first in a mixture of 2.5 μg/ml FL-phalloidin and 25 μg/ml of Concanavalin A conjugated to tetramethylrhodamine isothiocyanate. (TMRITC-Con A; prepared as described for TMRITC-BSA in reference 16) for 15 min at 25°C. For some experiments a δ-rhodaminylphalloidin (rhodaminyl-lysine-phallotoxin [RLP]) (19) was used at a con-

[1] *Abbreviations used in this paper:* DAPI, 4',-6 diamidine-2-phenylindole dihydrochloride; FL-phalloidin, fluorescein derivative of phalloidin; TMRITC-Con A, Concanavalin A conjugated to tetramethylrhodamine isothiocyanate.

centration of 1 μg/ml. This was found to be a stronger fluorochrome than FL-phalloidin because of its slower bleaching rate. In these experiments fluorescein isothiocyanate-Concanavalin A (FITC-Con A) (Miles Laboratories, Inc., Elkhart, IN) was used at a concentration of 25 μg/ml. After washing in PBS the nuclei were stained with 0.5 μg/ml 4',-6 diamidine-2-phenylindole dihydrochloride. (DAPI Boehringer Mannheim Biochemicals, Indianapolis, IN) in PBS for 10 min at 25°C. After several more rinses in PBS the embryos were mounted in 80% glycerol made up with 0.1 M Tris buffer pH 9.0. To observe both sides of the embryo, we mounted embryos individually between two coverslips (no. 0 Chance-Propper). To support the embryos, we placed a length of thin copper wire (0.2-mm-diam), on either side of them prior to the coverslips being glued together. The coverslips were then mounted on 0.8-1.0-mm-thick slides.

To check for nonspecific staining, we incubated embryos with an excess of unlabeled phalloidin (5 mg/ml) for 45 min at 25°C before and after staining with the fluorescent derivative as described by Warn and Magrath (17). This was done for both FL-phalloidin and δ-rhodaminylphalloidin. Only a slight increase in nonspecific background labeling was observed in both cases. No staining of the structures specifically stained by FL-phalloidin alone occurred. Some autofluorescence of the yolk granules inside the egg occurred but this did not interfere with observations of the caps. In addition, if the LP-450 filter was removed, some autofluorescence of the nuclei was noted when the DAPI was omitted.

Epifluorescence Microscopy: A standard R Zeiss microscope was used equipped with filter sets for both fluorescein and rhodamine. Photographs of the FL-phalloidin staining were taken with a LP-450 longwave pass filter in the condenser. This was then removed to allow excitation of the DAPI. Photographs of nuclei were taken after a few seconds of illumination such that fluorescence due to fluorescein had been bleached out. Photographs were taken with Trix-X pan film with exposure times of 2-10 s.

Staging of Embryos: The exact stages were determined by counting the number of nuclei down the length of the embryo and also from nuclear size, as described in (21).

RESULTS

Prior to the migration of the bulk of the nuclei close to the surface, the embryo was covered with an array of microprojections. These appear as a dense fluorescent meshwork when the plasmalemma was stained with TMRITC-Con A (Figs. 1 a, left and 2 a). Immediately beneath this in embryos counterstained with FL-phalloidin is a corresponding mesh of F-actin (Fig. 1 b, left and 2 b). This actin is closely associated with the plasmalemma. As the nuclei migrated closer to the surface a major change occurred in the organization of the cytoplasm and plasmalemma above them. A very dense meshwork of F-actin fibres formed above each nucleus. Within it some small aggregates could be seen. Initially, this meshwork was quite small but it grew rapidly as can be seen in Fig. 1 b, where newly appearing caps are present in the center and rapidly growing caps to the right. A higher power view of the forming actin-rich caps is seen in Fig. 3 b. Compared with Fig. 2 b, where caps have not yet started to form, a much denser mass of F-actin is present above each nucleus and the density of staining between each cap is reduced. By focusing up and down, the layer of cap F-actin was found to have some thickness. The actin seems to extend from beneath the plasmalemma to not far from the nuclei. In Fig. 3 c the bases of the actin meshworks surround faintly the prominent interphase nuclei. F-actin is also present between the caps as a layer containing denser irregular aggregates (Fig. 3 b). This layer runs into the material of the forming caps. As the caps formed, the surface plasmalemma bulged out. With TMRITC-Con A staining they appeared as dark areas surrounded by many folded microprojections that showed bright fluorescence (Fig. 3 a).

After formation the surface bulges went through four division cycles. Their behavior was very similar for all four cleavages. However, in stages 12 and 13, following the nomenclature of Zalokar and Erk (21), many more caps were
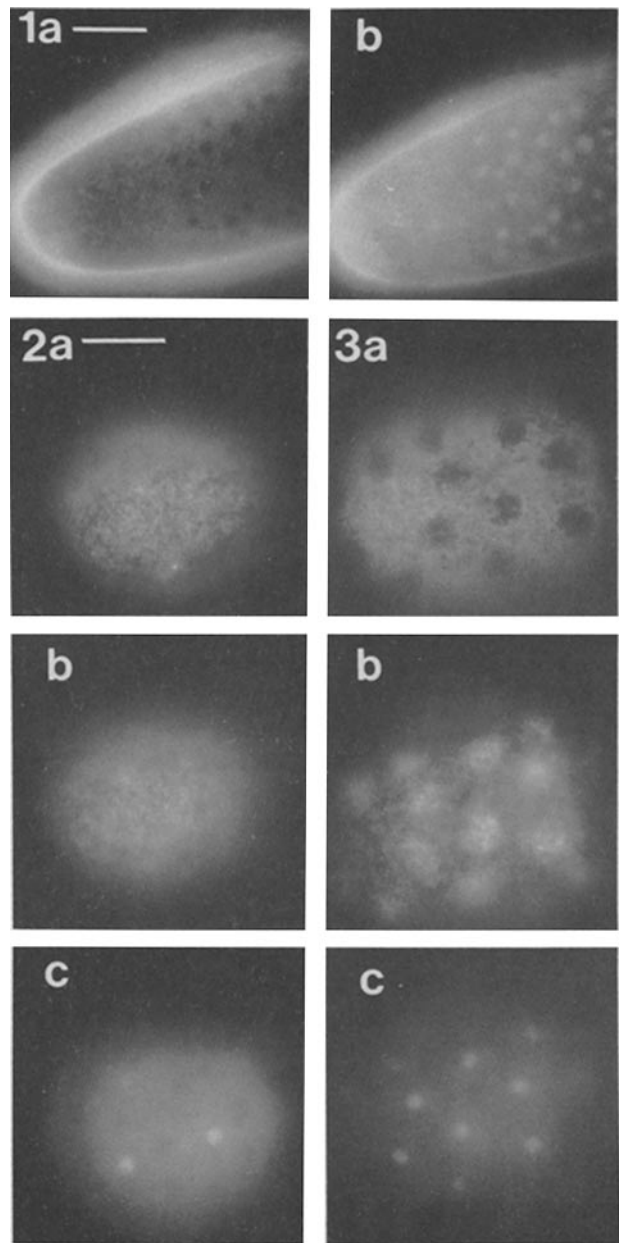
FIGURES 1-3   Fig. 1: Anterior half of an embryo where caps have formed in the middle portion but not yet at the anterior pole. (a) TMRITC-Con A fluorescence. (b) Same field as a but FL-phalloidin fluorescence. Bar, 60 μm. Figs. 2 and 3: Higher magnification of anterior pole (2, a-c) and middle section (3, a-c) of embryo of Fig. 1. (a) TMRITC-Con A. (b) FL-phalloidin. (c) DAPI. (Lettering in Figs. 4, 5, 7, and 9-13 refers to the same fluorescence optics as in Figs. 2 and 3.) Bar, 20 μm.

present and considerable interactions occurred between adjacent caps. The pattern of changes in cap structure will be described for stage-11 embryos. Where necessary it will be compared with the later stages.

At the beginning of the cycle the caps were irregular structures surrounded by varying amounts of membrane not included in the cap structure. Con A staining of the plasmalemma was not strong but it reveals a crinkled surface to the caps with Con A-positive folded edges (Fig. 4 a). Between the caps a number of small blebs are clearly visible. In fluorescein excitation optics the dense plaque of F-actin underlying the
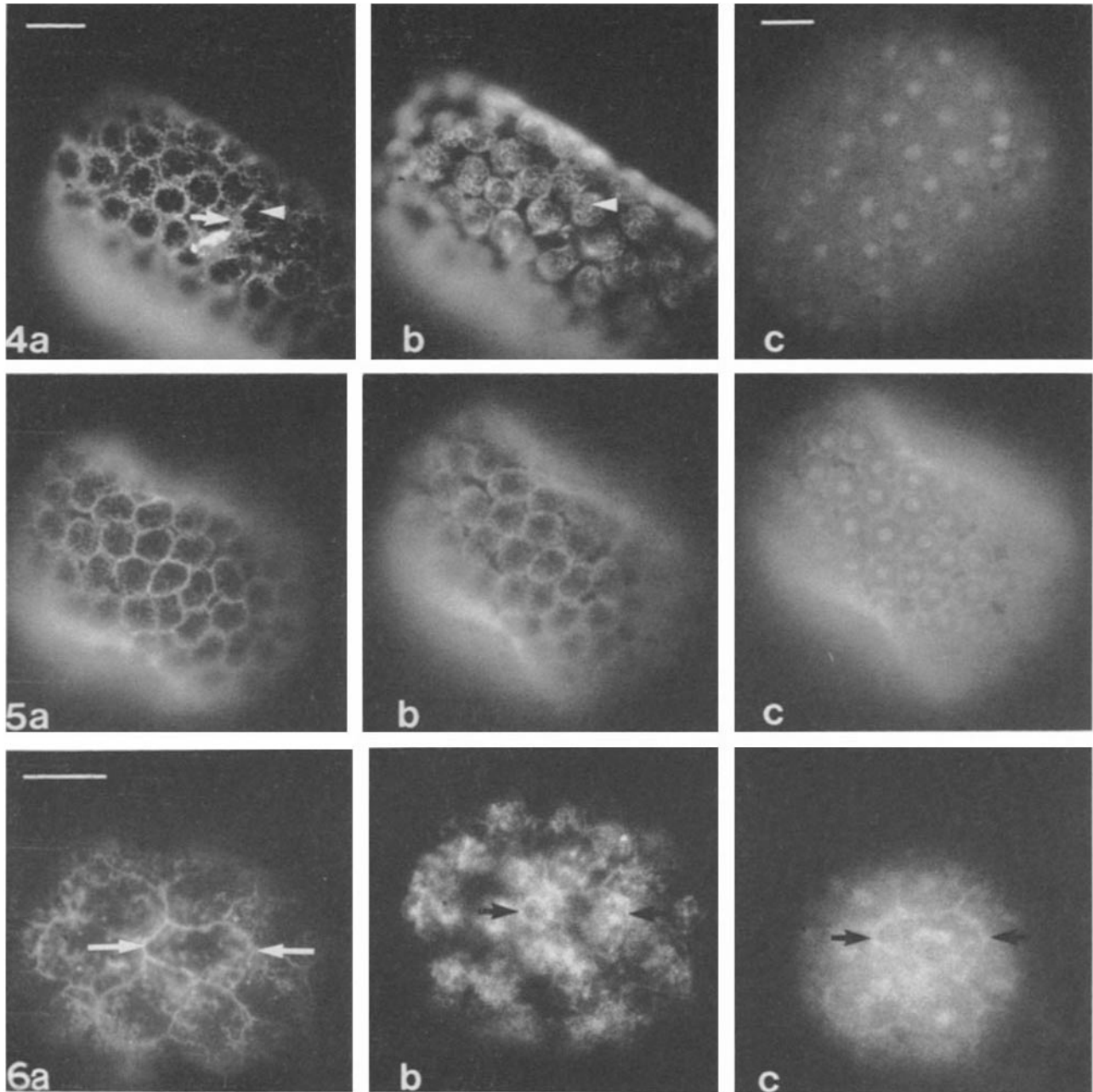
FIGURES 4–6   Fig. 4: Stage-11 blastoderm with caps beginning to expand. Arrow in *a* indicates blebs between caps. *c* shows a higher magnification of the nuclei for a different area of the same embryo as *a* and *b*. Arrowhead in *a* and *b* indicates fold underlain by strong F-actin staining. (*a* and *b*) Bar, 40 μm. (*c*) Bar, 20 μm. (Same magnification for Figs. 5 and 7.) Fig. 5: Stage 12, caps extending. Fig. 6, *a–c*: Stage 11, caps fully extended. Arrows indicate cap. *a* shows FITC-Con A fluorescence; *b*, same field with RLP fluorescence; *c*, DAPI. Bar, 20 μm. (Same magnification for Figs. 8–12.)

plasma membrane of each cap can be seen (Fig. 4 *b*). It consists of a discrete actin layer with distinctly brighter bands or folds within it. In some cases these clearly underlie the folds of the plasmalemma. In others, this is not apparently so. However, small changes of focus altered the pattern of observed F-actin. Some FL-phalloidin-staining material also underlies the blebs but, in comparison with the caps, the areas between them contain much less fibrous actin. The nuclei are in interphase and at maximum size. They stained rather diffusely with

DAPI (Fig. 4 *c*). Fig. 5, *a–c* is of a stage 12-embryo at a similar phase fairly early on in the cleavage cycle. The organization of the caps is much the same except that in Fig. 5 they are smaller and with a less pronounced folding of the plasmalemma. The spaces between caps are reduced and small blebs are not apparent. The organization of the F-actin layer of the caps is also much the same as in earlier stages except that the folding is less conspicuous.

By the end of the expansion process the caps had enlarged
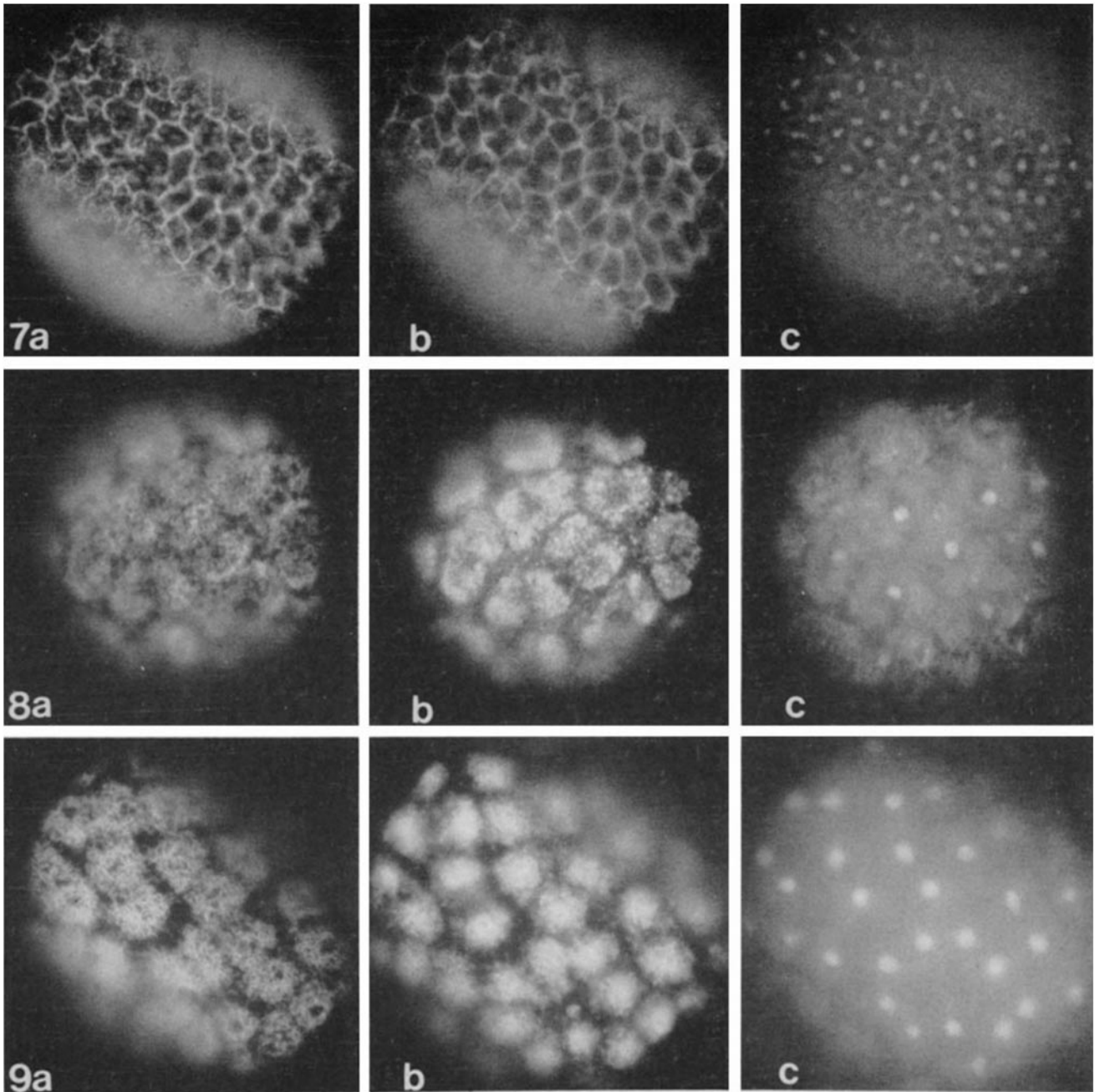
FIGURES 7–9   Fig. 7: Stage 13, caps fully extended. Fig. 8: Stage 11, caps in division. *a* shows FITC-Con A fluorescence; *b*) same field with RLP fluorescence; *c* DAPI. Fig. 9: Stage 11; daughter caps separating.

laterally to take in almost all the surface plasmalemma and abut each other (Fig. 6a). Between adjacent caps there is a distinct line of Con A staining that is underlain by a line of increased phalloidin staining (Fig. 6b). The surface of the caps is thrown into fine folds. These are more visible around the edges of Fig. 6a where the field of focus is at a slightly higher level than 6b. F-actin is rather more concentrated at the poles, with a much weaker area of staining in the center. The nuclei are in division by this time. In Fig. 6c they can be seen to be in anaphase. Fig. 7, a–c shows a stage-13 embryo where the caps have reached their maximum size and flattened out. All the caps are in close contact. In general, the structure was similar to that described for stage 11, except that the Con A staining between the caps was underlain by a more distinct

F-actin band. The caps at top left in Fig. 7b are somewhat further advanced than the others and show increased staining at both poles as described for Fig. 6b.

Figs. 8, 9, and 10 show the process of division of the caps. In Fig. 8 they are in the process of splitting. They are also beginning to bulge out again. The caps now show a much more convoluted surface with FITC-Con A staining than when very flattened. The cap surface area has become reduced but elevated in height so that regions between caps are visible again. In the center of Fig. 8b two caps show a central split with a narrow line of separation down the middle. Above these two more caps appear to be in the process of splitting. A central area is depleted in actin whereas the poles show strong phalloidin staining with small brighter aggregates pres-
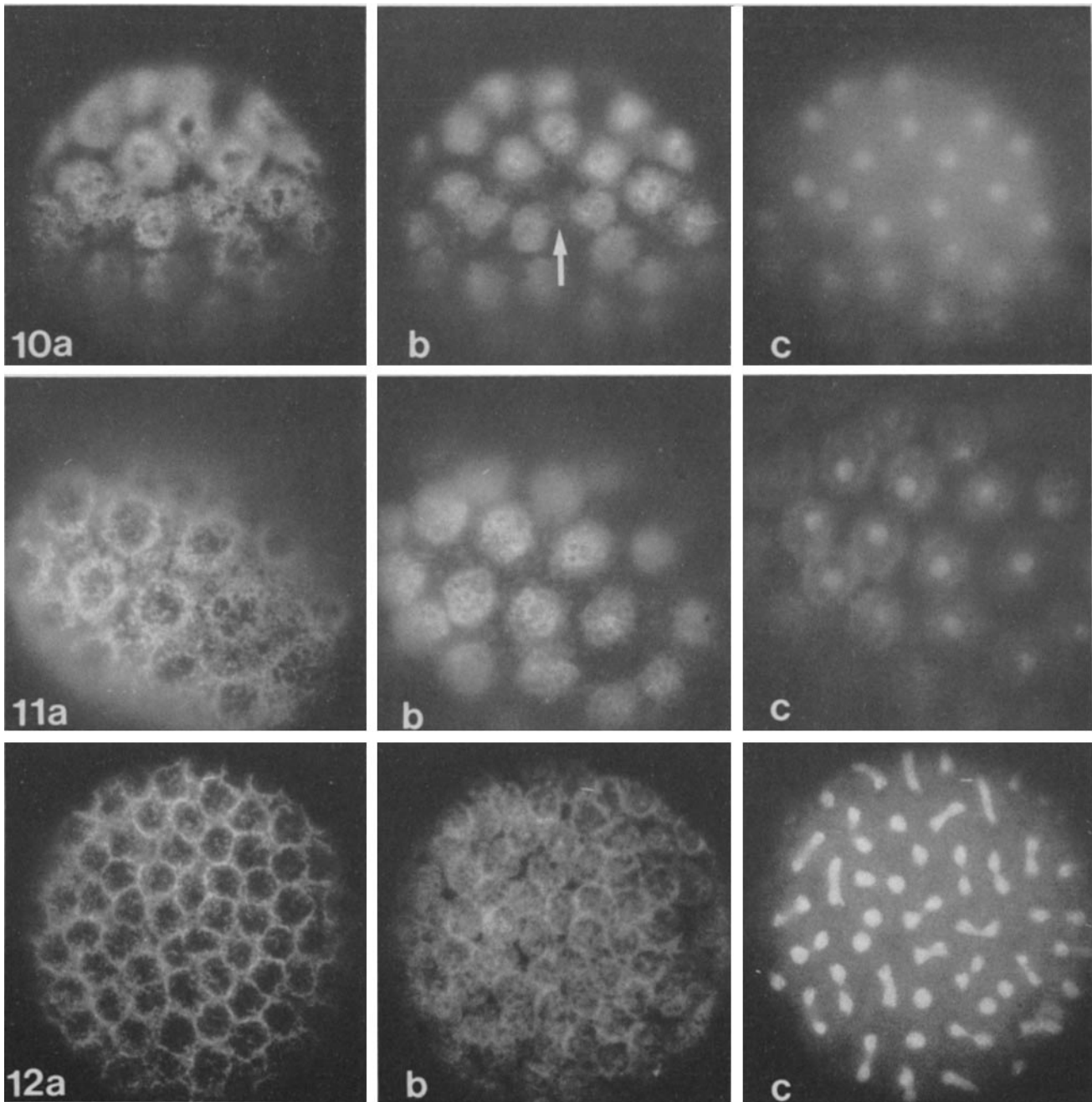
FIGURES 10–12   Fig. 10: Stage 11, caps reforming. Arrow in *b* indicates region between presumed daughter caps. Fig. 11: Stage 11, caps reformed. Fig. 12: Stage 13, caps reformed.

ent within the mesh. Apart from these central areas there has been a significant increase in F-actin density within the caps. At the edges brightly staining threads of actin are attached to the forming caps. In this embryo the nuclei are not yet divided, being in late prophase or early metaphase (Fig. 8c). We have found that the process of mitosis is not always synchronous with that of cap cleavage. For example, the caps in Fig. 6 are uncleaved whilst the nuclei are in anaphase. In contrast, the embryo in Fig. 8 has caps in cleavage whilst the nuclei are not yet divided. Fig. 9 shows a slightly more advanced embryo. The cap membranes are brightly stained with TMRITC-Con A and highly convoluted except for some small areas at the top (Fig. 9a). In comparison, areas between caps show little staining except for small processes that run back into the caps.

The surface between caps appears rather flat. Underneath the plasmalemma of the caps is a dense mesh of F-actin with bright dots of material occurring at the edges (Fig. 9b, which is at a slightly lower level of focus than Fig. 9a). Fine F-actin threads run from the caps. These underlie the Con A-staining processes. The new daughter caps are distinct and appear to be rounding up. Some F-actin is present between the daughter caps and underlies membrane stained with Con A in this region. It is, however, much more diffuse than the actin within the new caps. Other areas between caps show little F-actin. Beneath, the nuclei are in telophase, except a pair at top left in Fig. 9c which are in late anaphase. Fig. 10, a–c shows further separation of the caps coupled with a rounding up and loss of the processes radiating from them. In Fig. 10a the

membranes over the caps are still very convoluted and brightly stained except for small dark regions at some of the apices. The F-actin cortical layers of the caps now have clearly defined edges and the majority of the processes extending from them have disappeared (Fig. 10 b). There is still some FL-phalloidin staining between daughter caps but this is reduced and diffuse. Nuclei are now in interphase (Fig. 10 c).

By the end of cleavage each cap had become entirely separate again. The surfaces of the caps now appear rather dark with only a few Con A-staining folds or projections (Fig. 11 a). However, the edges of the caps stained strongly for Con A and appear to be thrown into many folds. In the regions between caps a number of Con A-staining blebs are now evident. Beneath the surface the layer of actin associated with each cap has fully reformed (Fig. 11 b). The structure now resembles that of the caps at the start of the previous cycle (Fig. 4) except that it is smaller in size. In the regions between caps some F-actin is present, apparently associated with the microprojections of the plasmalemma. Underneath the caps the nuclei are in interphase (Fig. 11 c). A basically similar organization is found for the caps of the stage-13 embryo shown in Fig. 12, a–c. The plasmalemma of the caps is not as highly convoluted and only a number of small folds can be seen (Fig. 12 a). However, the membrane around the edges is brightly fluorescent and shows fine folding. Underneath, each cap is marked out by a dense meshwork of F-actin. Within the meshwork some areas of stronger FL-phalloidin staining occurred but it has not been possible to determine any clear relationship with the plasmalemma folding. Around the edges fairly bright FL-phalloidin staining is present in regions where TMRITC-Con A fluorescence is also strong. Increased F-actin at the margins of the caps is more characteristic of the later stages where the caps are crowded together. The nuclei underneath the caps are in anaphase or telophase even though the caps have divided (Fig. 12 c). In this case the asynchrony between the two processes is obvious.

DISCUSSION

In any fluorescence microscopy study it is essential to make sufficient controls to check the specificity of the staining reagents. We have previously demonstrated that insect F-actin but not G-actin blocks the staining by FL-phalloidin, as does mammalian F-actin (17). Because of this specificity for F-actin (18, 20), FL-phalloidin has been found to be superior to antiactin antibodies in double-labeling experiments (14). Staining reactions due to the fluorochrome and also autofluorescence may present problems. Another control is to block staining of actin by incubation with a large excess of unlabeled phalloidin before and after staining with the fluorescent phalloidin. In this way any binding due to the fluorochrome can be detected. At the syncytial blastoderm stage, only a slight increase in general background fluorescence was found after blocking with unlabeled phalloidin. The only specific autofluorescence seen was from the yolk and some from the nuclei when the longwave pass filter was removed. Phalloidin can also enter cells fixed with formaldehyde alone. Stronger agents such as alcohols, acetone, or detergents are usually required to allow the entry of antibodies into cells. Such agents may damage or alter the organization of the cytoskeleton in situ.

A second question relevant to any histological preparation is how far the fixed material resembles the structural organization in vivo. Formaldehyde is recognized to be a relatively mild fixing agent but we have noticed some flattening and compression of the embryo during fixation. Because of this it is necessary to select caps least affected by compression, particularly for the stages following division when the caps protrude most. Glutaraldehyde fixation is better in this respect but much increases the autofluorescence. A further problem is the presence of the impermeable vitelline membrane which must be removed. The choice of the method of removal has been discussed elsewhere (17).

The use of FL- and δ-rhodaminyl phalloidin has shown the caps above the superficial blastoderm nuclei to be highly organized structures. They form as the result of a reorganization of an actin-rich cortical meshwork that underlies the plasmalemma of the preceding nuclear multiplication stage. The arrival of the nuclei to within a few microns of the surface was followed by the formation of the caps. Whether the initiation of cap formation caused a tighter packing of F-actin above each nucleus or promoted further polymerization in this region is not known. The two possibilities are not exclusive. At the same time, a significant diminution was found in the staining density of the cap plasmalemma by fluorescently labeled Con A. The change in Con A surface membrane labeling closely followed the enlargement of the actin web of the caps and may suggest some functional relationship. However, surface cap formation is accompanied by the reorganization of the many microprojections into which the plasmalemma was previously folded (13). Presumably, the bulging out of the caps incorporates the membrane foldings. This reduction and change in folding could also at least partially explain the apparent reduction in staining of the plasmalemma of the forming caps.

Once formed, the F-actin webs of the caps go through cyclic patterns of changes correlated with cap expansion and division. Whilst the caps expand they squeeze and push round each other so that almost the whole embryo surface is included in the caps prior to division (16). These movements are more evident in the third and fourth blastoderm cleavages when the surface is more crowded with caps. Such cap movements have also been recorded in the blastoderm of Callosobruchus embryos (7). The expansion of the caps is closely related to the extension of the F-actin meshwork and it may be that this meshwork is the principal agent causing expansion.

There is a further role for the actin meshwork in holding the nucleus within the cap as it divides. In experiments where permeabilized Drosophila blastoderm stages were treated with cytochalasin B the usual regular pattern of divisions became disrupted (21). Anaphase and telophase nuclei of adjacent caps were found colliding into each other. At the next division polyploid figures occurred, presumably resulting from multiple nuclei dividing within the same cap. These results correlate well with the observed structure of the F-actin layer of the caps, particularly in the later blastoderm stages prior to division where there was a significantly denser layer of actin at the edges. It appears likely that the actin meshwork acts in some way to constrain the spindle apparatus and prevent the division products from leaving the cap as it divides. As a result each nucleus becomes associated with a daughter cap.

The failure to find any significant band of actin microfilaments associated with the cleavage zone of the caps was surprising. Ultrastructural evidence from various groups, both vertebrate and invertebrate, has demonstrated the ubiquity of this structure in the animal kingdom (Reviews 3, 9). Usually a complete ring of microfilaments is found within the cleavage furrow. However, in the eggs of Loligo (1, 2), the colenterates Aequorea (12), and Stomatoca (8), cleavage is restricted to the animal pole of the early embryo and does not completely

separate the blastomeres. Microfilaments are present as a band beneath the plasma membrane of the cleavage furrow. It has been suggested that they divide the blastomeres by the band of filaments shortening whilst the plasmalemma extends (1, 2). At the outset of this study it seemed likely that some similar structure would occur, but in none of the material has any sign been seen of a band of F-actin along the axis of cleavage. Rather, a marked diminution of staining intensity was found in this region. It could be argued that within the cleavage zone there is a special group of microfilaments, presumably associated with myosin, which has not been detected. There is debate as to whether or not the contractile ring is distinguishable in all types of cultured cells after staining with antiactin antibodies or fluorescent heavy meromyosin (cf. 3 for a review). In all studies with one exception this has been the case. So far, fluorescently labeled phalloidin has not been used to study F-actin distribution in cleaving cultured cells. During the cellularization of the *Drosophila* blastoderm a strongly staining hexagonal network can be found at the bases of the extending plasmalemmas using FL-phalloidin (17). These results and other evidence (5, 21) suggest that the F-actin network acts as a series of interlocked contractile networks pulling down all the cell membranes simultaneously. From these data, we think it likely that FL-phalloidin would also identify F-actin within a contractile ring structure of the surface caps at the syncytial blastoderm stage, if it existed.

If a band of filaments was to cut through a cell, then the cell surface must be curved so that the contractile band can shorten whilst pulling down the plasmalemma. Observations of the movements of *Drosophila* blastoderm caps have been made by microinjection of TMRITC-labeled BSA into the space between the vitelline membrane and the embryo (16). Under epifluorescence microscopy the surface caps are visible by a kind of negative contrast against the red of the soluble protein that fills the space. As the caps change shape and height the relative intensity of the surrounding fluorescence alters. By these means it can be seen that the blastoderm caps are virtually flat immediately prior to division. A re-examination of the data has found that, when a separation between daughter caps occurs, the intensity of the dye remains approximately the same as that in the grooves around the caps. The daughters form as hemispherical bulges that rapidly darken against the background fluorescence. These results suggest that there is no cutting into the embryo cortex by a cleavage furrow as new caps form. Rather, the caps bubble out from the surface. In contrast, the *Loligo*, *Aequorea*, and *Stomatoca* eggs are all spherical.

In regular cells cytokinesis is accompanied by inward movement of significant amounts of membrane. But little total change in membrane area accompanies cap division for they are only shallow protuberances. The special requirements of orienting nuclei close to the surface in a syncytium appear to have given rise to a specialized alignment mechanism where the cap itself behaves as an expanding plate of microfilaments.

At present there is little direct evidence concerning an alternative mechanism by which cleavage of the caps might occur. So far, two points have been established. Firstly, prior to division an increased F-actin network became apparent at both poles and the area between them became depleted in actin. Secondly, when division occurred the daughter caps were strongly stained by FL-phalloidin, demonstrating a high concentation of F-actin to be present. It may be that the forming daughter caps rupture the old cap plate by a contrac-

tion of the actin networks associated with the two poles. This would result in an increased density of the F-actin associated with each new cap and also increase the folding of the surface membrane as was observed. Antibody staining has shown the presence of myosin within the caps (15) and it will be of interest to determine the distribution of myosin in the region of cap cleavage. The small FL-phalloidin-staining processes attached to the new caps could represent part of the contraction of the polar actin into the new daughter caps. The result of the contraction and rounding up of the polar material would be to form a protrusion on the surface. This new cap would then start to expand and take in new surface membrane as the underlying actin network began to relax and extend. On this model the F-actin plate acts somewhat like a net which is extended, ruptured into two, and then the two halves drawn in. It may be that the structure revealed by fluorescence microscopy has a molecular organization similar to that of the networks of short bifurcated actin filaments present in the cortex of cranefly spermatocytes (11). These filamentous arrays markedly resemble those found in erythrocytes. Such a structure would be likely to have the kind of properties required for the suggested molecular organization of the blastoderm caps.

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