An Investigation of Microtubule Organization and Functions in Living *Drosophila* Embryos by Injection of a Fluorescently Labeled Antibody against Tyrosinated α-Tubulin

R. M. Warn, L. Flegg, and A. Warn

School of Biological Sciences, University of East Anglia, Norwich, NR4 7TJ, United Kingdom

Abstract. Rhodamine-labeled monoclonal antibodies, which react with tyrosinated α-tubulin (clone YL 1/2; Kilmartin, J. V., B. Wright, and C. Milstein, 1982, *J. Cell Biol.*, 93:576–582) and label microtubules in vivo (Wehland, J., M. C. Willingham, and I. Sandoval, 1983, *J. Cell Biol.*, 97:1467–1475) were microinjected into syncytial stage *Drosophila* embryos. At 1 mg/ml antibody concentration, the microtubule arrays of the surface caps became labeled by YL 1/2 but normal development was found to continue. The results are compared with the data from fixed material particularly with regard to interphase microtubules, centrosome separation, and spindle and midbody formation. At 5 mg/ml antibody concentration the microtubules took up larger quantities of antibodies and clumped around the nuclei. Nuclei with clumped microtubules lost their position in the surface layer and moved into the interior. As a result, the F-actin cap meshwork associated with such nuclei either failed to form or subsided. It is concluded that microtubule activity is required to maintain the nuclei in the surface layer and organize the F-actin meshwork of the caps.

I N recent years the introduction of fluorescently labeled proteins and other probes into cells, combined with the use of image intensification cameras and enhancement techniques, has permitted the visualization of cell organization in vivo in molecular terms (for reviews see Taylor and Wang, 1980; Kreis and Birchmeier, 1982). The method has a number of advantages over the examination of fixed material, which it complements. Artefacts due to fixation can be detected by comparing the results obtained with those found with different fixation protocols. The time course of events can be directly determined. More significantly continuous observation can reveal rare or highly transitory phenomena which might otherwise be missed.

The microtubule network has been much studied by this method, mainly using a variety of tubulin derivatives (Keith et al., 1981; Wadsworth and Sloboda, 1983). This work has demonstrated that fluorescently labeled tubulin becomes incorporated into microtubules with very fast kinetics, suggesting that microtubules are much more dynamic structures than had been previously suggested. Further elegant work has determined the rates of microtubule elongation, both for interphase arrays (Saxton et al., 1984; Soltys and Borisy, 1985; Schulze and Kirschner, 1986) and also for the mitotic spindle (Salmon et al., 1984 *a, b*; Mitchison et al., 1986).

Up to now few studies have followed the changing microtubule distribution over longer time courses; e.g., early development where the cytoskeleton undergoes continuous changes during the rapid cell cycles. However, Hamaguchi et al. (1985) have followed the distribution of fluorescently labeled brain tubulin in fertilized sea urchin eggs and demonstrated that it integrated into the microtubules of the pronuclear asters and then into the mitotic spindle. The changing tubulin distribution could be followed throughout the first and subsequent cleavages.

An alternative method for labeling microtubule arrays is to use antitubulin antibodies. Of particular interest is the anti-α-tubulin monoclonal antibody YL 1/2 developed by Kilmartin et al. (1982). This antibody recognizes the carboxy-terminal tyrosinated form of α-tubulin (Wehland et al., 1983, 1984).

Wehland et al. (1983) and Wehland and Willingham (1983) have microinjected this antibody into cultured cells. At low concentrations (2 mg/ml in the injected solution) no obvious effects on microtubule behavior were seen. Saltation continued suggesting normal intracellular transport was occurring as did cell locomotion. But at higher concentrations (6 mg/ml) these processes were blocked and microtubule bundling occurred within the cell. Using still higher concentrations (12 mg/ml) the microtubule bundles were found to collapse around the nuclei. Thus, using YL 1/2, it should be possible to label microtubules in a noninhibitory manner at low concentrations and also to block their activity at higher concentrations to allow a functional investigation.

The *Drosophila* embryo is suitable material for in vivo studies being readily available and easy to microinject. Furthermore, the embryos are very tolerant of continued illumination with the fluorescence microscope, even in the absence of filters that reduce the light intensity (Warn and Magrath,

1982). By comparison cultured cells are much more light sensitive (our unpublished observations). The embryo develops very rapidly with the first 13 divisions completed within 2.5 h (Zalokar and Erk, 1976; Foe and Alberts, 1983). During these divisions the embryo is a syncytium. After 10 cleavages, most of the nuclei come to lie within 5 µm of the surface and the syncytial blastoderm is formed. The behavior of the nuclei is therefore easily observable by epifluorescence microscopy during this stage. The nuclei become enclosed within protrusions of the plasmalemma and associated cortical cytoplasm forming cell-like surface caps. Each cap is associated with a well-developed cytoskeleton including microtubules, F-actin microfilaments, and intermediate filaments (see Warn, 1986). The caps then go through four further mitotic cycles before cells form simultaneously over the whole surface of the embryo during the cellular blastoderm stage.

In this paper we describe the labeling pattern of rhodamine YL 1/2 injected into embryos at low and higher concentrations, and examine its effects upon the cap cytoskeleton.

Materials and Methods

Collection, preparation, and microinjection of embryos were carried out as described previously (Warn and Magrath, 1982). The only change to the techniques was that the syringe, tubing, and pipette shaft were filled with 3S Voltalef oil (Ugine Kuhlman, Paris, France) rather than paraffin oil. Embryos were usually injected 10–20 min before observations to allow sufficient diffusion of the antibodies. The precise cap cycle number was determined by counting back from when cellularization was first obvious. Micropipettes with an external diameter of 15 µm were used and 0.15–0.3 nl of liquid were introduced into the center of each embryo midlaterally. To ensure that the same volume of material was introduced each time, the amount of fluid drawn into the pipette was measured with an eyepiece graticule. All experiments were done at an ambient room temperature of 20 ± 2°C.

In Vivo Observations

Observations were made with an Intensified Silicon Intensified Target (ISIT) image intensification camera (model TC 1040/H; RCA, Lancaster, PA) mounted on top of a Universal microscope (Carl Zeiss, Inc., Thornwood, NY). To enhance the image it was processed through a "Crystal" digital processing system (Quantel, Surrey, U.K.). A X40 plan-neofluar lens (Carl Zeiss, Inc.) was used, immersed directly into 10S Voltalef oil in which the embryos were immersed. For higher magnification, an X4 adaptor tube (Carl Zeiss, Inc.) was added between the camera and the microscope. Recordings were made on a "U-matic" V.T.R. (Sony, Tokyo, Japan). Photographs were taken directly from the monitor (Melford, U.K., long persistance model). The rat monoclonal YL 1/2 were gifts of Dr. J. Wehland (Max-Planck Institute, Gottingen, FRG) and Dr. J. Kilmartin (Laboratory of Molecular Biology, Cambridge). It was labeled with rhodamine (rh)' using TRITC as described previously (Wehland and Willingham, 1983).

Fixation and Counterstaining of Embryos

Embryos were fixed in 8% paraformaldehyde, 0.5 µM taxol in MES (2-[N-morpholino]ethane sulfonic acid) buffer (100 mM MES, 1 mM EGTA, 1 mM Mg SO₄, pH 6.9). The fixative was added directly on top of the embryos in Voltalef oil. After 30 min, the vitelline membranes were dissected off with fine needles and the embryos removed and washed. F-actin microfilaments were then stained with fluorescein (fl)- or rh–phalloidin derivatives and nuclei stained with DAPI as described previously (Warn et al., 1984; Warn et al., 1985) except that the nuclei were visualized by excitation in the far UV and observed in the blue emission wavelengths (Zeiss filter set 01). Embryos were mounted in 90% glycerol containing 2.5% propyl gallate as an antiquenching agent.

1. *Abbreviations used in this paper*: fl, fluorescein; rh, rhodamine.

Results

Microinjection of YL 1/2 at 1 mg/ml

10–20 min after the microinjection of ∿0.2 nl of rh-YL 1/2 into embryos it became associated with the microtubule arrays of the cleaving nuclei. Although the antibody continued to diffuse towards the embryo poles until the cellular blastoderm had been formed it was possible to make continuous observations of these microtubule networks during the repeated mitotic cycles of the syncytial blastoderm and also during the process of cellularization and beyond (not shown). Embryos thus treated continued to develop normally during at least an hour or more of continuous illumination and then carried on through development. Generally such embryos developed into unhatched larvae with varying degrees of abnormality. However, a small proportion of them (∿5%) hatched (Fig. 1, *a* and *b*). In general, more embryos hatched from batches where the smallest quantities of antibody had been injected.

The fluorescence present in such larvae was mainly inside the gut as had previously been found for rhodamine-labeled BSA (rh-BSA) similarly injected (Warn and Magrath, 1982). But rather more fluorescence was noted in other tissues than had been seen with rh-BSA and this raised the possibility that functional YL 1/2 was still present inside larval cells. However, we have not seen any clearly labeled spindles present in the larvae. The majority of these larvae developed normally into adults.

Tubulin Distribution during the Mitotic Cycles of the Syncytial Blastoderm as Revealed with rh-YL 1/2

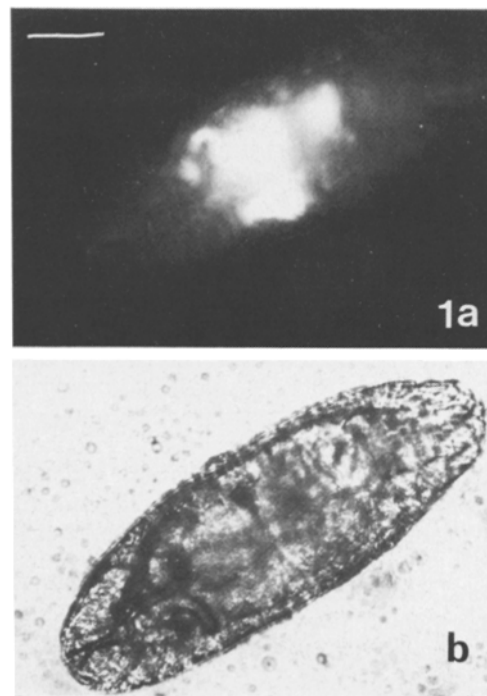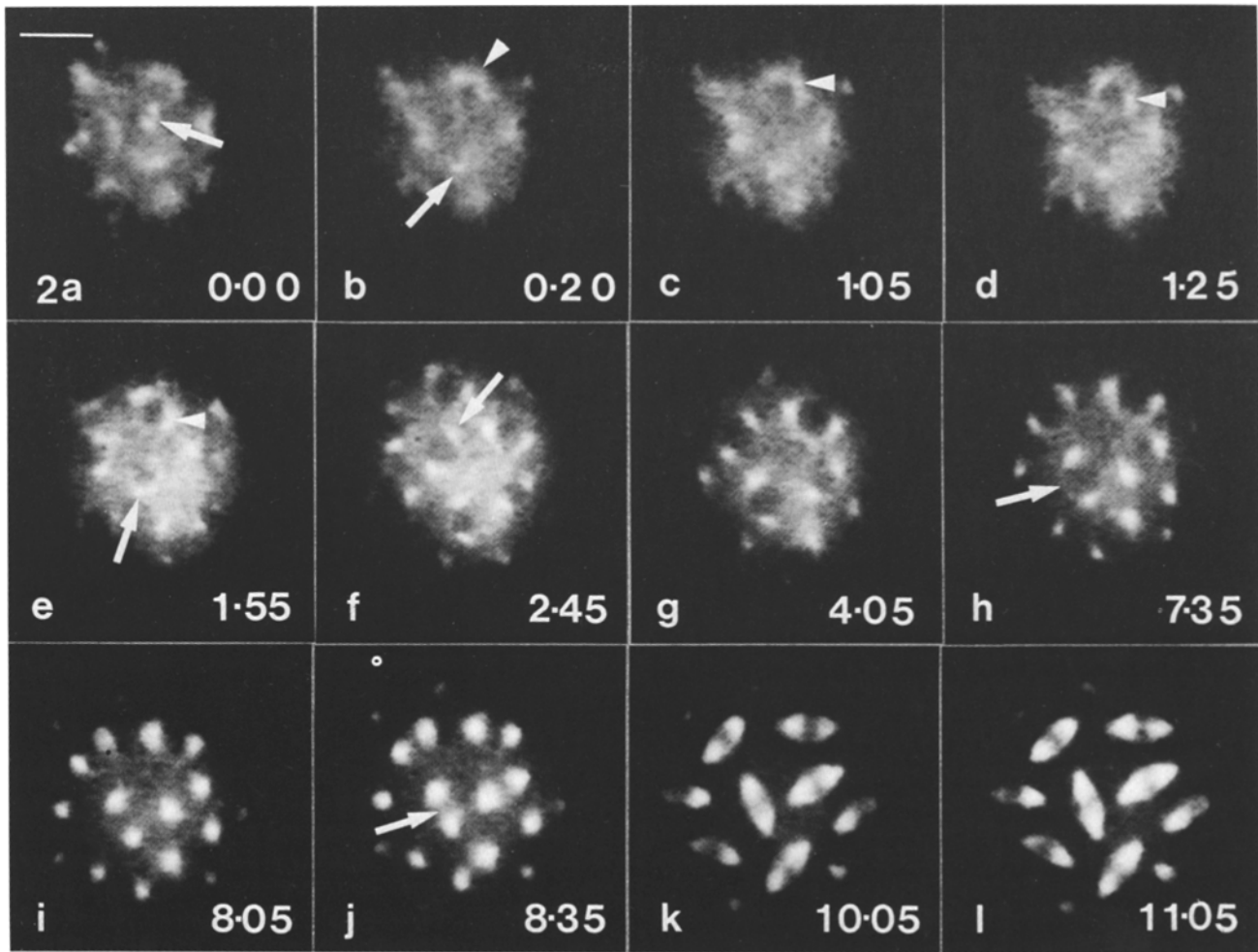At higher magnification it was possible to follow the chang-

Figure 1. Embryo injected with rh-YL 1/2 (1 mg/ml) at late preblastoderm which has developed to hatching. (*a*) Fluorescence distribution; (*b*) phase micrograph. This larva developed into a normal adult. Bar, 70 µm.

Figure 2. Series of micrographs from monitor to show the changing rh-YL 1/2 distribution in surface caps from the end of telophase 11 to metaphase 12. Time given in minutes and seconds from the first micrograph. (*a*) (*Arrow*) Midbody. (*b*) (*arrowhead*) Fluorescent crescent marking the asters; (*arrow*) centrosome migrating in lower cap. (*c–e*) (*Arrowhead*) Centrosome migrating around side of nucleus. (*e* and *f*) (*Arrows*) Centrosomes migrating back into plane of focus. (*h*) (*Arrow*) Outline of nucleus. (*j*) (*Arrow*) Microtubule growth between the centrosomes. Bar, 10 μm.

ing tubulin distribution during the mitotic cycle. This was best done during the syncytial blastoderm stage when the nuclei were stably associated with the plasmalemma in the cell-like caps, rather than the preceding preblastoderm stage (when the nuclei were migrating towards the surface). Our results were very similar for all the four mitotic cycles of the syncytial blastoderm stage, except for the size of the spindles which was reduced for each successive cycle.

Interphase was judged to start when the midbody disappeared and the astral fibers became the interphase microtubule array (Fig. 2, *a* and *b*). Individual microtubules were usually hard to identify but a bright fluorescent mass filled the area of the reforming caps, marking the interphase array. Fluorescence was brightest as a crescent around the nuclei, marking the position of the former asters. What were presumed to be the locations of the centrosomes were first visible as somewhat brighter areas within the fluorescence adjacent to the nuclei (Fig. 2, *b* and *c*). Frequently the centrosomes moved out of focus to a position above the nucleus soon after the end of telophase (e.g., lower cap; Fig. 2 *b*). However, on occasion the centrosomes did not move upwards

but could be observed migrating around the sides of the nuclei towards the sites of the future spindle poles (e.g., upper cap in Fig. 2, *c–e*). In caps where the centrosomes migrated above the nucleus they returned to the same level of focus as the asters of the previous spindles, either simultaneously or nearly together (Fig. 2, *e* and *f*).

By this time the caps had entered prometaphase. This phase was marked by a rapid build-up of tubulin around the centrosomes, as shown by YL 1/2 fluorescence (Fig. 2, *e–h*). During the development of bright fluorescence in the pericentrosomal region, the fluorescence in other regions of the caps gradually became reduced as the interphase array of microtubules was lost. While the build-up of fluorescence at the spindle poles continued the outlines of the nuclei were still prominent as shown by their encirclement with fluorescence (Fig. 2 *h*). Then, rapidly, the outline of the nuclei became indistinct (Fig. 2 *i*) and microtubules grew between the poles to create the spindles (Fig. 2 *j*). While the spindles formed, the fluorescence at the poles was incorporated into them (Fig. 2, *i–l*). The cytoplasmic fluorescence also became considerably reduced while the spindles developed, in-
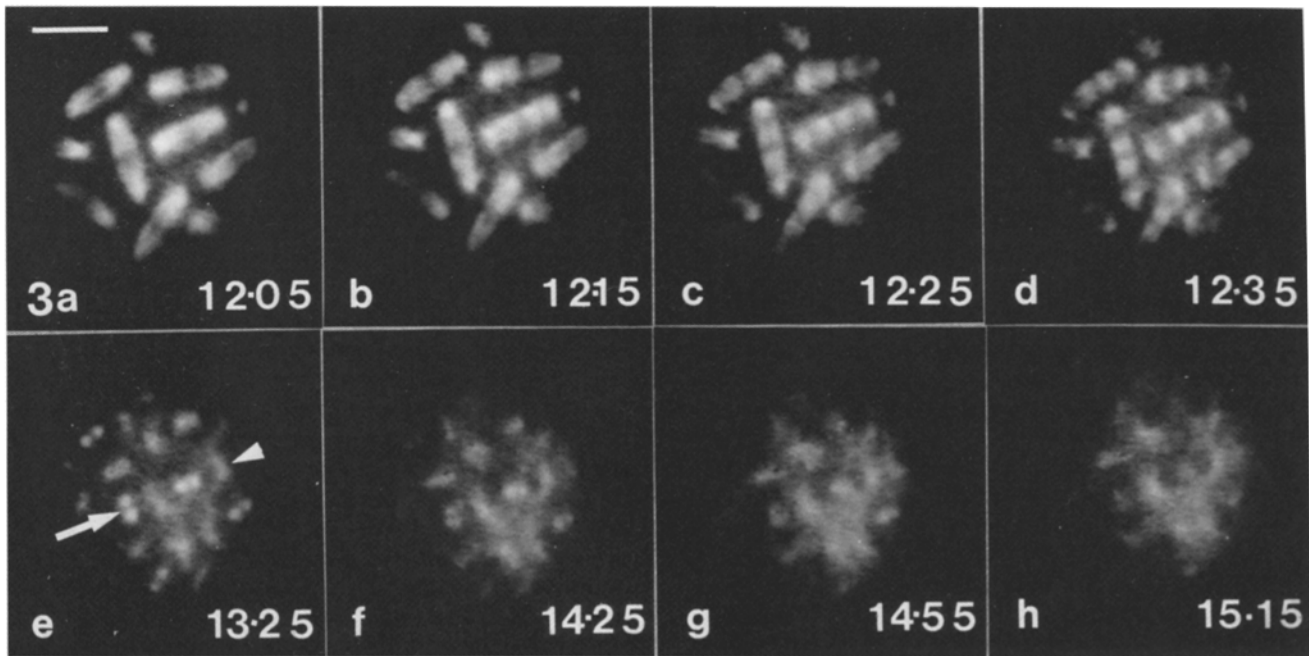
Figure 3. Continuation from Fig. 2 showing YL 1/2 distribution during anaphase and telophase of cycle 12. The midbody and astral microtubules are indicated by the arrow and arrowhead, respectively, in *e*. Bar, 10 μm.

dicating that much of the tubulin was incorporated into the spindles. Only very small asters were visible during metaphase. A central region of reduced fluorescence was present within the spindles (the interzone) but with some microtubules running across this region (Fig. 2, *k* and *l*).

Anaphase was found to be marked by a further lengthening of the spindle together with the appearance of distinct poles (Fig. 3, *a* and *b*). The spindle assumed a more rectangular shape. As the poles became more conspicuous during anaphase, microtubules started to grow from them developing into distinct asters (Fig. 3, *b–d*).

The caps then passed into telophase. This stage was accompanied by the loss of kinetochore microtubules and the emergence of a prominent midbody between the reforming daugher nuclei (Fig. 3, *d* and *e*). The midbody was made up from the interzone region including the central area of reduced fluorescence, which was present throughout the chromosome movements of anaphase. When the midbody formed the intensity of fluorescence within it increased relative to the brightness around it in the spindle. It then rapidly reduced in size (Fig. 3, *f* and *g*). This loss of material occurred over the whole of the midbody. Simultaneously the nuclei gradually became visible again against the fluorescent background, presumably because of the exclusion of tubulin from the nucleus. The midbody finally disappeared completely at the end of the mitotic cycle leaving the daughter nuclei surrounded by crescents of brighter fluorescence within a distinct background of fluorescence (Fig. 3 *h*).

Cap Cycle Length

It was possible to time the phases of the cap cycle for the four mitoses of the syncytial blastoderm stage. Using this method the length of each cycle has been found to be as follows (figures in brackets give the range of variation): cycle 10 (11 min [8.5–12.5 min]); cycle 11 (12 min [9–11 min]); cycle 12

(14.5 min [11.5–18 min]); cycle 13 (20 min [13.5–29 min]) (20 embryos examined). Each cycle was found to be a little longer than the previous one and cycle 13 rather more so. There was quite a wide variation in cycle length between embryos; i.e., some embryos having shorter cycles than others.

In addition, times were recorded for various phases of the four cell cycles of syncytial blastoderm. The data is given in Table I. The start of each cycle was timed from the final disappearance of the midbody. Because it was difficult to observe the separation of the centrosomes, it was more accurate to measure the time when they arrived at the poles to start prometaphase. Therefore, interphase and prophase were included together. Similarly, prometaphase and metaphase could not be separated. However, anaphase was a quite distinctive and rapid event, lasting about a minute or less. Telophase began with the appearance of the midbody between the reforming nuclei.

In general, there was an increase in all parts of the cycle as successive cycles lengthened. This lengthening was much more marked for the phases before anaphase. Interphase + prophase and prometaphase + metaphase roughly doubled in length from cycles 10–13. In contrast, anaphase and telophase showed only small mean increases in length.

As would be expected from the variation in cycle lengths there was quite a variation in lengths for different parts of the cycle. Embryos with longer or shorter phases for all parts of successive cycles were found quite frequently. Occasionally successive cycles had phases that were shorter than the same phase in the preceding cycle.

Microinjection of YL 1/2 at 5 mg/ml

Injection of ∼0.3 nl of antibody but at five times the concentration gave very different results. The microtubule arrays associated with the nuclei became very brightly stained, taking up larger quantities of antibody than at 1 mg/ml. Frequently

Table I. Phase Lengths of Different Parts of the Four Mitotic Cycles in Drosophila Syncytial Blastoderm Stages

Stage of development	Interphase + prophase	Prometaphase + metaphase	Anaphase	Telophase
Cycle 10 (6)	3.7 (1.5–6.0)	4.7 (3.75–6.0)	0.5 (0.5–0.66)	2.1 (1.0–3.5)
Cycle 11 (9)	3.3 (2.0–6.0)	5.8 (5.0–7.0)	0.5 (0.5–0.75)	2.4 (1.0–3.5)
Cycle 12 (13)	4.1 (2.5–9.5)	7.8 (4.75–11.5)	0.66 (0.5–1.0)	2.1 (1.0–4.0)
Cycle 13 (13)	7.4 (3.25–14.75)	9.0 (6.75–15.0)	0.8 (0.5–1.25)	2.4 (1.5–5.0)

Average durations and range (in parentheses) of the various phases in the syncytial blastoderm mitotic cycles as determined from playback of tapes. Times are given in minutes. Numbers of embryos observed are given in parentheses for each cycle.

In quite a proportion, the embryos were seen to have mitotic gradients present. The times were therefore determined for one particular part of the embryo.

the nuclei attempted to undergo mitosis and bizarre spindles were formed (Fig. 4 *a*). The spindles clumped as nonfunctional fluorescent masses. In addition, small dotlike structures were often present. These were presumably asters which had detached from the spindles because occasionally very elongated spindles were seen with very similar dot-like asters at their ends. Such spindles did not divide and subsequently sunk into the interior of the embryo.

In embryos given half the volume of YL 1/2 at 5 mg/ml the loss of nuclei from the surface layer occurred only around the region of injection. Spindles in regions further away continued to divide and some of these moved into the regions where functional spindles had been lost. An example of what was observed is given in Fig. 4, *b–g*. In Fig. 4 *b*, a number of YL 1/2-stained spindles were visible in the central regions of the field. They failed to go into anaphase unlike the one at the base (Fig. 4, *b* and *c*). By the prophase of the next division, functional spindles had moved up into the bottom region of the illuminated zone and during this mitosis the inactive spindles in the central region had sunk away from the cortex into the interior or were in the process of doing so. As these spindles moved into the more central region the inactive spindles were also pushed along by the advancing stream. (Compare the arrows in *b* and *d*). In addition, a number of detached asters were visible ahead of the dividing

spindles (Fig. 4 *d*). Usually they appeared to be single and were presumed to have detached from nuclei which had sunk out of the cortical layer. However, they were seen on occasion in pairs as miniature spindles (not shown). These minispindles have been observed to elongate during anaphase and attempt to divide.

The areas formerly occupied by inactive microtubule arrays can become completely taken over by functional microtubule arrays associated with nuclei moving in from the sides. Fig. 4 *e* shows the further migration of spindles into the unoccupied region by the metaphase of the following division. All the inactive spindles had disappeared into the interior. Some small aster-like structures were still visible – associated with the advancing front of nuclei. In this embryo the movement of microtubule arrays into the unoccupied region was continuous and this was the case for a number of other embryos similarly injected. However, we have noted other embryos injected with identical amounts of YL 1/2 where periodic movements of the embryo occurred towards and away from the region of injection during the mitotic cycle in similar fashion as was previously described by Foe and Alberts (1983). In Fig. 4 *f* the functional spindles can be seen to have spread over the whole of the central region and no inactive microtubule arrays were finally visible. In this embryo both this mitosis and the previous one showed marked
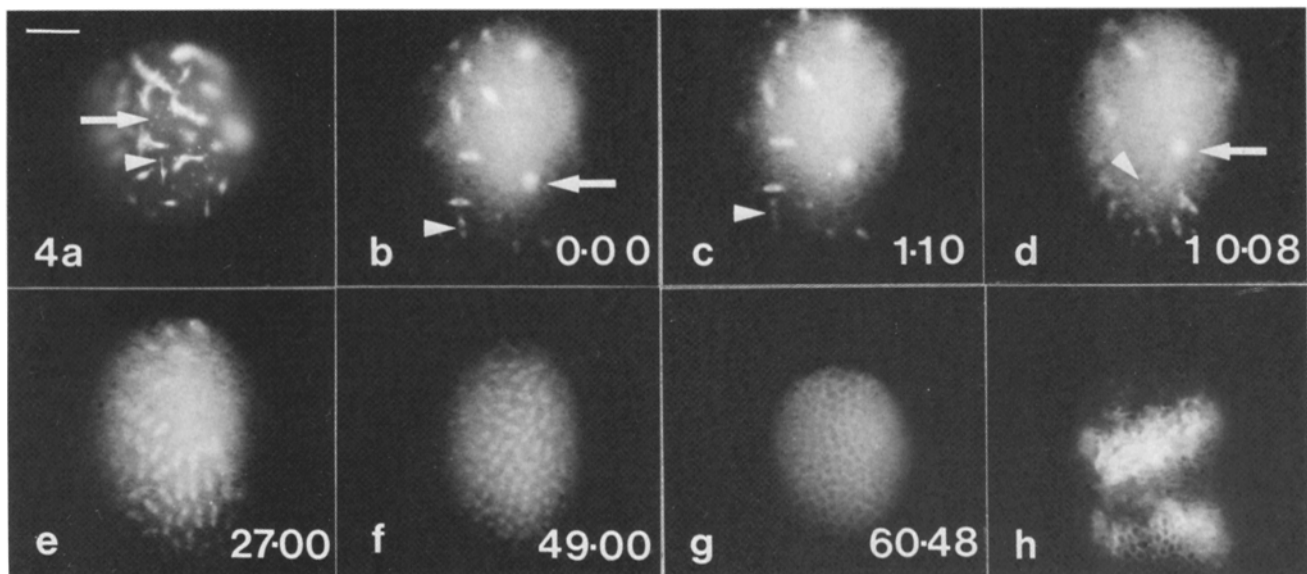
Figure 4. (*a*) Embryo injected with 5 mg/ml rh-YL 1/2 at preblastoderm and observed 30 min later at syncytial blastoderm. (*Arrow*) Detached asters; (*arrowhead*) aster still attached to elongated spindle. (*b–g*) Series of micrographs to show loss of inactive spindles and repopulation by migration from other areas. (In *f* and *g*, the field of focus has been narrowed somewhat.) (*b* and *d*) (*Arrow*) Inactive spindle. (*b* and *c*) (*Arrowhead*) Elongating spindle; (*d*) (*arrowhead*) isolated asters. (*h*) Embryo injected as in *a* and allowed to develop to cellular blastoderm. The upper area was the site of injection. Bar, 20 μm.
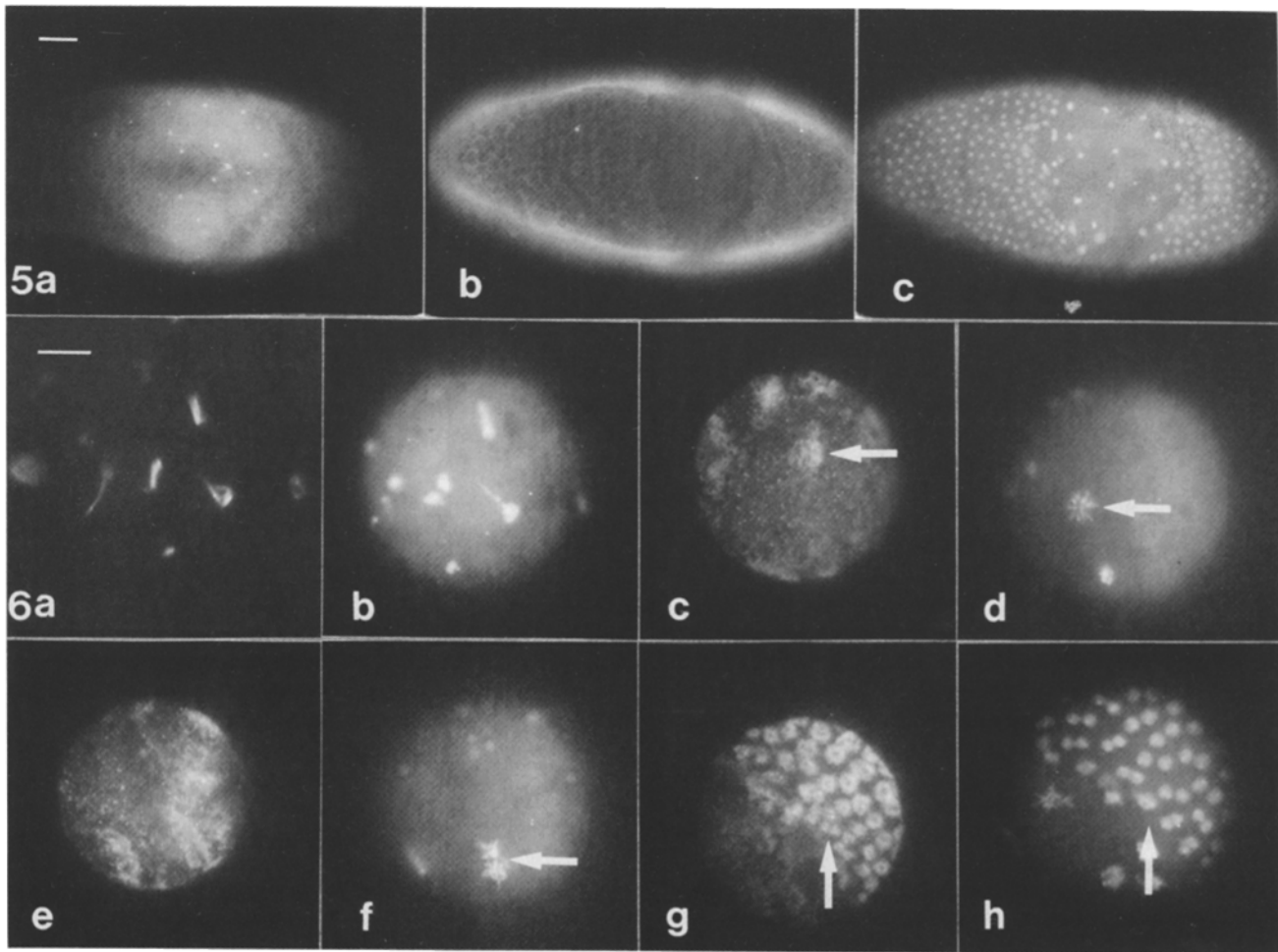
Figures 5 and 6. (Fig. 5) Embryo injected with 5 mg/ml rh-YL 1/2 at preblastoderm and allowed to develop for a further 30 min. (*a*) rh-YL 1/2; (*b*) fl-phalloidin; (*c*) DAPI. Bar, 10 μm. (Fig. 6, *a–f*) High power micrographs of embryos injected as in Fig. 5. (*a*) rh-YL 1/2. (*b*) DAPI counterstain. (*c*) rh-phalloidin of similar region of another embryo; arrow indicates abnormal cap lacking nucleus. (*d*) DAPI counterstain; arrow indicates nucleus isolated from any cap. (*e*) Central region with no caps obvious. (*f*) DAPI counterstain; arrow indicates isolated nucleus. (*g* and *h*) Embryo injected in cycle 10 or 11 and allowed to develop 40 min before fixation: (*g*) rh-phalloidin; (*h*) DAPI counterstain. Arrows indicate minicaps lacking nuclei. Bar, 40 μm.

mitotic gradients. Thus in Fig. 4 *f* the spindles at the top were in metaphase while those at the bottom were in prophase. In a number of cases we have seen that the spindles closest to the inert cortical areas divided ahead of regions further away. This embryo, like a number of others formed a seemingly normal cellular blastoderm with microtubules around the nuclei (Fig. 4 *g*) and continued development beyond gastrulation. But in other cases the microtubule arrays which formed during cellularization were disorganized in the region where inactive spindles were present (Fig. 4 *h*). Cells did not form in the disorganized region. In no case did normal larvae form after microinjection of rh-YL 1/2 at 5 mg/ml.

To investigate further what happened to the caps after injection of rh-YL 1/2 at the higher concentration some embryos were injected either before or during the syncytial blastoderm stage, allowed to develop for varying periods of time, and then fixed and stained with rh- or fl-phalloidin and DAPI. Fig. 5, *a–c*, shows a low power view and Fig. 6, *a–h*, shows high power micrographs of embryos thus treated. Brightly stained clumps of microtubules were found associated with abnormal nuclei present in the central region of the embryo, where they were previously injected (Figs. 5 *a*, 6, *a* and *b*). Quite a significant amount of general bright fluorescence was also present in this region; this was either antibody bound to tubulin monomers or unbound antibody. Fl-phalloidin staining (Figs. 5 *b* and 6, *c–f*) demonstrated that caps had either failed to form or only partially formed if preblastoderm stages were microinjected. Over large areas only an irregular cortical network of F-actin was present (Fig. 6 *e*). Included in it were the usual numerous small F-actin aggregates. At varying depths into the interior abnormal, irregular mitotic figures were present, showing varying degrees of clumping (Fig. 5 *c* and Fig. 6, *b*, *d*, and *f*). If early syncytial blastoderm stages were microinjected the caps which received larger amounts of antibody were found often to have largely subsided (Fig. 6 *g*, bottom left).

Embryos which had been allowed to develop for a longer period after injection usually showed smaller abnormal zones (Fig. 7, *a–c*). This finding correlates with the data already described for living embryos; i.e., movement of caps occurs into regions where nuclei have sunk into the interior. Such embryos often had areas with small caps at the bound-
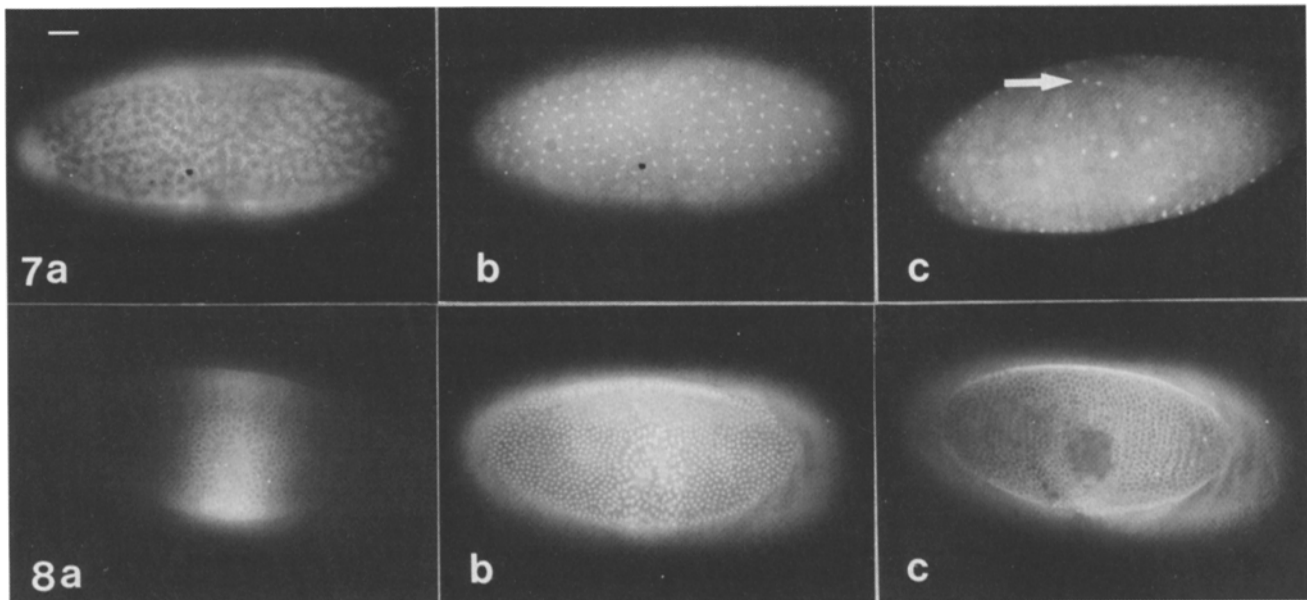
Figures 7 and 8. (Fig. 7) Embryo injected as in Fig. 5 but allowed to develop 45 min before fixation. (*a*) rh-phalloidin; (*b*) DAPl at same level of focus as in *a*; (*c*) (*arrow*) nuclei presumed to have moved out of surface layer (slightly lower level of focus than in *b*). (Fig. 8) Embryo injected during early syncytial blastoderm and allowed to develop to cellular blastoderm stage. (*a*) rh-YL 1/2; (*b*) DAPl; (*c*) fl-phalloidin. Bar, 35 μm.

ary with the region lacking caps (Fig. 6, *g* and *h*). These minicaps frequently did not contain nuclei. In addition, larger caps lacking nuclei were also seen in the boundary zone.

Embryos that were allowed to continue development to the cellular blastoderm stage often contained areas which had failed to cellularize (Fig. 8, *a–c*). These embryos failed to make the normal perinuclear microtubule arrays in the region where antibody had been previously injected (Fig. 8 *a*) and contained abnormal nuclei (Fig. 8 *b*). In these regions F-actin was present as a layer of diffuse material rather than organized as the system of hexagonal rings which surround each forming cell at the tip of the inward-growing membrane (Fig. 8 *c*).

Discussion

This paper demonstrates that if an appropriate concentration is used (1 mg/ml), rh-YL 1/2 is a satisfactory marker for the in vivo labeling of the microtubule networks in *Drosophila* embryos, which does not unduly disturb further development. In some cases larvae develop from the injected embryos and a proportion of them hatch and develop into adults. Furthermore, the evidence does not suggest that cleavage is significantly slowed as a result of YL 1/2 binding to the microtubules at this lower concentration because the timed average durations of cleavage are comparable with published data. Warn and Magrath (1982) and Foe and Alberts (1983) reported slightly faster average cleavage times for the four syncytial blastoderm cleavages as compared with those presented here (although Foe and Alberts made their measurement at 25°C), while Edgar et al. (1986) found comparatively slower rates for each cycle except cycle 10. The average time for cycle 13 reported here was also faster than that of Foe and Alberts (1983). The general conclusion from a comparison of these data is that the binding of the antibody to tubulin had no significant effect on the average rates of division.

Fidelity of Image

With any study involving a monoclonal antibody it is important to know what epitopes are shared with other proteins. For YL 1/2 the binding epitope sequence has been identified (Wehland et al., 1984). Apart from binding to ribonucleotide reductase (Standart et al., 1985), the available evidence suggests YL 1/2 only binds to tyrosinated α-tubulin (Wehland et al., 1983, 1984). Only tubulin-rich structures were visualized in this investigation. YL 1/2 has previously been shown to bind to *Drosophila* α-tubulin (Warn and Warn, 1986). Recently, the sequence of the four *Drosophila* α-tubulins has been determined (Theurkauf et al., 1986). Two of the three α-tubulins (α 1 and 3), which are present in early stage embryos, code for proteins very similar to previously sequenced α-tubulins and have a carboxy-terminal tyrosine residue. The third (α 4) has a rather different sequence and ends with a carboxy-terminal phenylalanine. At present it is not known whether YL 1/2 identifies α 4, although from the given sequences it presumably labels both α 1 and α 3 tubulin.

In this study individual microtubules were only rarely seen and in the absence of discrete microtubules the video images differ from those obtained by immunofluorescence staining of fixed material. This is not due to the image intensification camera because microtubules are readily visible with the same system after microinjection of rh-YL 1/2 into living cultured mammalian cells (Prescott, A., and R. M. Warn, unpublished results). The difference could be due to excess antibody not bound to α-tubulin in the *Drosophila* embryos. Against this argument the mitotic spindles were surrounded by a much lower background than interphase and other stages. Thus, it is much more likely that the interphase microtubule arrays were seen against a background of significant amounts of unpolymerized tubulin. Furthermore, the surface caps are rather small structures, only some 5–20 μm in diameter and contain rather small spindles and interphase arrays as compared with cultured cells.

Antibody Labeling of the Tubulin Pool

Does the endogenous tubulin pool remain constant within each cap during the mitotic cycles of the syncytial stages? In the experiments described above, the embryos were not filled with antibody. Only the middle portions of the embryos were brightly fluorescent and a fluorescent gradient existed to completely dark poles. This contrasted with the effects of microinjection of rh-BSA into embryos where it rapidly diffused throughout the embryos which became uniformly fluorescent (Warn and Magrath, 1982). In contrast, rh-YL 1/2 was much more restricted in its diffusion. However, it was seen to slowly diffuse through the embryos during the 1-h duration of syncytial blastoderm. We have found no evidence that clearly demonstrates bound antibody becoming compartmentalized within the caps, although this is possible.

Early-stage *Drosophila* embryos are very rich in maternally derived stores of α and β tubulin and it has been estimated that 10% of the nonyolk protein within the egg is tubulin (Loyd et al., 1981). In addition, there is a large store of maternally transcribed tubulin mRNA (Kalfayan and Wensink, 1982; Mischke and Pardue, 1982; Natzle and McCarthy, 1984). But it is not known whether these transcripts are translated during syncytial blastoderm, or whether tubulin degradation occurs, so the question of to what extent tubulin–antibody complexes cycle between the interphase and spindle microtubules cannot be addressed.

Comparison with Immunofluorescence Studies of Fixed Material

Interphase Microtubule Arrays. The distribution of tubulin in fixed early stage *Drosophila* embryos has been described by two groups using rather different procedures (Warn and Warn, 1986; Karr and Alberts, 1986). Several significant differences in the pattern of tubulin distribution are apparent upon comparing the results. With both protocols the mitotic spindles were well preserved and, in general, similar distributions were seen, although with methanol fixation rather slimmer spindles were found with less well-developed asters. However, during interphase greater differences between the two procedures were apparent. With methanol fixation only sparse microtubules were seen, radiating from pairs of prominent centrosome like bodies (Warn and Warn, 1986). In contrast, taxol/formaldehyde treatment resulted in caps with abundant microtubule arrays, but no discernible centrosomes (Karr and Alberts, 1986).

The data from in vivo observations give useful information to reconcile the contrasting data. Centrosomes were indeed visible as brighter areas within the fluorescence which marks the interphase and prophase microtubule arrays. It is concluded that both taxol/formaldehyde and methanol fixation would seem to create some fixation artefacts. It would appear that methanol clumps interphase microtubules during fixation allowing visualization of the position of the centrosomes. It also seems possible that taxol causes some polymerization of pericentrosomal tubulin monomers before fixation, thus causing the centrosomes to be obscured. A possible slight polymerization effect of taxol may also explain why spindles labeled with rh-YL 1/2 in vivo appeared rather more fusiform and with reduced asters as compared with taxol/formaldehyde-fixed material.

The in vivo rh-YL 1/2 observations suggest that centrosome separation is an early event in the mitotic cycle. Because separated centrosomes can be seen within a couple of minutes from the end of telophase, the centrioles are presumed to separate at an early point indeed in the mitotic cycle. This corresponds with findings from haematoxylin-stained embryos, where centrioles were found to have split by late anaphase ready for the next mitosis (Huettner, 1933). The separation of centrioles and centrosomes has generally been considered to be a feature of prophase so their early duplication is presumably a reflection of the very rapid separation rates in the early stages of development.

Spindle Formation. The above observations suggest that spindle formation occurs in two phases: a build-up of a ball of tubulin/antibody fluorescence at the poles, followed by a growth of microtubules to form the spindle. Immediately before the second phase of spindle formation, the nucleus becomes accessible to tubulin as judged by the loss of contrast compared with the cytoplasmic fluorescence. This is in agreement with the finding that spindle formation occurs within the nuclear envelope in *Drosophila* embryos with microtubules growing in from the ends (Stafstrom and Staehelin, 1984). It may be that the centrosomal region concentrates a store of tubulin monomers as the interphase array disassembles. These monomers are then reformed into the spindle microtubules. Alternatively, the fluorescent ball contains short microtubules which subsequently elongate into the nucleus.

Midbody Formation. One advantage of in vivo observations is that the formation of structures can be observed directly rather than reconstructed from fixed material of differing stages. Our observations show that the midbody forms from the interzone region of the spindle. This zone has its origins as the metaphase plate where the chromosomes are located (Warn and Warn, 1986). In embryos such as the sea urchin (Harris et al., 1980; Balczon and Schatten, 1983) and cultured cells (Weber et al., 1975; Fuller et al., 1975; Fujiwara and Pollard, 1978; Vandre et al., 1984), the midbody appears as a group of microtubules spanning the intercellular bridge remaining at the end of cytokinesis. The structure is clearly keyed into the interphase networks but a description of how it forms from the spindle is lacking for these cells.

Two possible models of the mechanism by which midbodies form are as follows: (*a*) they occur as the result of the partial breakdown of polar microtubules from the ends attached to the centrosomes; (*b*) new microtubules form which are not directly attached to the centrosomes. In the case of the *Drosophila* syncytial blastoderm spindles, the midbody microtubules are found to run from nucleus to nucleus and there is no evidence that they attach directly to the centrosomes (Warn and Warn, 1986). The present observations do not rule out either of the above hypotheses concerning midbody formation and further work is required to investigate the problem.

What might the function of the midbody be, particularly in a situation where cytokinesis does not occur? The caps are dynamic structures which expand and flatten, pushing against each other in the process (Warn and Magrath 1982; Foe and Alberts, 1983). We have noted several instances where rotation of daughter caps occurred at the time the midbody disappeared. Thus the midbody may hold the daughter caps to-

gether until the events of mitosis have been completed and the cap-splitting process is finished.

Effects of YL 1/2 at Higher Concentration

At a higher concentration (5 mg/ml), microtubules took up larger quantities of antibody and became nonfunctional. The results for *Drosophila* embryos correspond with those found for cultured cells by Wehland and Willingham (1983). In both cases microtubule activity was lost, the microtubules bundled around the nucleus, and mitosis was blocked.

Interactions between Microtubules and Cortical F-actin: The Organization of the Cap Domain

When nuclei and their associated microtubules were rendered inactive by YL 1/2 injected at the higher concentration, they were lost from the surface layer. This suggests that dynamic interactions occur between the microtubules and the cortical surface layer to maintain the nuclei within this layer. In regions where nuclei were lost, we found that the caps failed to form and when present they frequently subsided, or completely disappeared from the affected region, as judged by phalloidin staining. Microtubules are thus strong candidates for organizing both the formation and maintenance of cap structure. Caps are highly structured cytoplasmic domains and the distributions of microfilaments and microtubules within them correspond during interphase (Karr and Alberts, 1986). Treatment with cytochalasin B causes at least some loss of cap structural integrity (Zalokar and Erk, 1976). Thus the microtubule array may organize cap structure via the cortical F-actin network which delimits it (Warn et al., 1984; Warn, 1986; Karr and Alberts, 1986). There is some evidence in favor of the general hypothesis that microtubules can organize actin microfilaments, as in the case of the contractile ring at cell cleavage (Rappaport, 1971) and also in situations where changes in cell shape occur (Solomon and Magendantz 1981; Tomasek and Hay, 1984). Cap organization is a further example where a correlation exists between structural and functional interactions for microtubules and microfilaments. Whether interactions also exist between microtubules and intermediate filaments within the caps (cf., Walter and Alberts, 1984) remains to be investigated.

Lateral Cap Migration

The lateral movement of new caps into regions where others have subsided or failed to form occurs as the result of movements within the embryo. These movements would appear to be similar to those related to the mitotic cycle (Kinsey, 1967; Foe and Alberts, 1983) although we frequently found them to be continuous rather than restricted to various phases. The significance of this difference is not clear at present. While the caps move laterally there must be progressive remodeling of the surface. Material not included in the caps is presumably incorporated at the advancing cap edges and lost at the trailing edges. Replacement of caps in the middle region of the embryo has not been reported before but mutations exist where nuclei do not migrate directly into the posterior region but do so by lateral migration of the caps (Niki and Okada, 1981; Niki, 1984).

As the caps move they push before them the remains of caps rendered nonfunctional. Frequently, very small caps

are found in this region. These are most probably associated with isolated centrosomes, which remain connected with the cortical layer. Examination of fixed uninjected material has occasionally revealed groups of such minicaps (Warn, 1986). In the maternal effect mutant *gnu* centrosome cleavage becomes uncoupled from nuclear division (Freeman et al., 1986). In this mutant the free centrosomes migrate independently to the surface and initiate aster formation but it is not known whether minicaps also form as a result of centrosome migration to the cortical region.

In conclusion, microinjection of rh-YL 1/2 has demonstrated the existence of very dynamic tyrosinated microtubule arrays which are closely associated with cortical actin structures. The molecular mechanisms regulating the assembly and disassembly kinetics of these microtubules, and their interactions with the cortex remain to be elucidated.

References

Balczon, R., and G. Schatten. 1983. Microtubule-containing detergent extracted cytoskeletons in sea urchin eggs from fertilization through cell division: antitubulin immunofluorescence microscopy. *Cell Motil.* 3:213–226.

Edgar, B. A., C. P. Kiehle, and G. Schubiger. 1986. Cell cycle control by the nucleo-cytoplasmic ratio in early *Drosophila* development. *Cell.* 44:365–372.

Foe, V. E., and B. Alberts. 1983. Studies of nuclear and cytoplasmic behaviour during the five mitotic cycles that precede gastrulation in *Drosophila* embryogenesis. *J. Cell Sci.* 61:31–70.

Freeman, M., C. Nusslein-Volhard, and D. M. Glover. 1986. The dissociation of nuclear and centrosomal division in *gnu*, a mutation causing giant nuclei in *Drosophila*. *Cell.* 46:457–468.

Fujiwara, K., and T. D. Pollard. 1978. Simultaneous localization of myosin and tubulin in human tissue culture cells by double antibody staining. *J. Cell Biol.* 77:182–195.

Fuller, G. M., B. R. Brinkley, and J. H. Boughter. 1975. Immunofluorescence of mitotic spindles by using monospecific antibody against bovine brain tubulin. *Science (Wash. DC).* 187:948–950.

Hamaguchi, Y., M. Toriyama, H. Sakai, and Y. Hiramoto. 1985. Distribution of fluorescently labelled tubulin injected into sand dollar eggs from fertilization through cleavage. *J. Cell Biol.* 100:1262–1272.

Harris, P., M. Osborn, and K. Weber. 1980. Distribution of tubulin containing structures in the egg of the sea urchin *Strongylocentrotus purpuratus* from fertilization through first cleavage. *J. Cell Biol.* 84:668–679.

Huettner, A. F. 1933. Continuity of the centrioles in *Drosophila melanogaster*. *Z. Zellforsch. Mikrosk. Anat.* 19:119–134.

Kalfayan, L., and P. C. Wensink. 1982. Developmental regulation of α-tubulin genes. *Cell.* 29:91–98.

Karr, T. L., and B. M. Alberts. 1986. Organization of the cytoskeleton in early *Drosophila* embryos. *J. Cell Biol.* 102:1494–1509.

Keith, C. H., J. R. Feramisco, and M. Shelanski. 1981. Direct visualization of fluorescein-labeled microtubules *in vitro* and in microinjected fibroblasts. *J. Cell Biol.* 88:234–240.

Kilmartin, J. V., B. Wright, and C. Milstein. 1982. Rat monoclonal antitubulin antibodies derived by using a new nonsecreting rat cell line. *J. Cell Biol.* 93:576–582.

Kinsey, J. D. 1967. Studies on an embryonic lethal hybrid in *Drosophila*. *J. Embryol. Exp. Morphol.* 17:405–423.

Kreis, T. E., and W. Birchmeier. 1982. Microinjection of fluorescently labelled proteins into living cells with emphasis on cytoskeletal proteins. *Int. Rev. Cytol.* 75:209–227.

Loyd, J. E., E. C. Raff, and R. A. Raff. 1981. Site and timing of synthesis of tubulin and other proteins during oogenesis in *Drosophila melanogaster*. *Dev. Biol.* 86:272–284.

Mischke, D., and M. L. Pardue. 1982. Organization and expression of α-tubulin genes in *Drosophila melanogaster*. *J. Mol. Biol.* 156:449–466.

Mitchison, T. J., L. Evans, E. Schulze, and M. Kirschner. 1986. Sites of

microtubule assembly and disassembly in the mitotic spindle. *Cell.* 45: 515–527.

Natzle, J. E., and B. J. McCarthy. 1984. Regulation of *Drosophila* α and β-tubulin genes during development. *Dev. Biol.* 104:187–198.

Niki, Y. 1984. Developmental analysis of the grandchildless (gs (1) N26) mutation in *Drosophila melanogaster*: Abnormal cleavage patterns and defects in pole cell formation. *Dev. Biol.* 103:182–189.

Niki, Y., and M. Okada. 1981. Isolation and characterization of *grandchildless*-like mutants of *Drosophila melanogaster*, *Wilhelm Roux's Arch. Dev. Biol.* 190:1–10.

Rappaport, R. 1971. Cytokinesis in animal cells. *Int. Rev. Cytol.* 31:161–213.

Salmon, E. D., W. M. Saxton, R. J. Leslie, M. L. Karow, and J. R. McIntosh. 1984a. Diffusion coefficient of fluorescein-labeled tubulin in the cytoplasm of embryonic cells of a sea urchin: a video image analysis of the fluorescence distribution after photobleaching. *J. Cell Biol.* 99:2157–2164.

Salmon, E. D., R. J. Leslie, W. M. Saxton, M. L. Karow, and J. R. McIntosh. 1984b. Spindle microtubule dynamics in sea urchin embryos: analysis using a fluorescein-labeled tubulin and measurements of fluorescence redistribution after laser photobleaching. *J. Cell Biol.* 99:2165–2174.

Saxton, W. M., D. L. Stemple, R. J. Leslie, E. D. Salmon, M. Zavortink, and J. R. McIntosh. 1984. Tubulin dynamics in cultured mammalian cells. *J. Cell Biol.* 99:2175–2186.

Schulze, E., and M. Kirschner. 1986. Microtubule dynamics in interphase cells. *J. Cell Biol.* 102:1020–1031.

Solomon, F., and M. Magendantz. 1981. Cytochalasin separates microtubule disassembly from loss of asymmetric morphology. *J. Cell Biol.* 89:157–161.

Soltys, B. J., and G. G. Borisy. 1985. Polymerization of tubulin in vivo: direct evidence for assembly onto microtubule ends and from centrosomes. *J. Cell Biol.* 100:1682–1689.

Stafstrom, J. P., and A. Staehelin. 1984. Dynamics of the nuclear envelope and of nuclear pore complexes during mitosis in the *Drosophila* embryo. *Eur. J. Cell Biol.* 34:179–189.

Standart, N. M., S. J. Bray, E. J. George, T. Hunt, and J. V. Ruderman. 1985. The small subunit of ribonucleotide reductase is encoded by one of the most abundant translationally regulated maternal RNAs in clam and sea urchin eggs. *J. Cell Biol.* 100:1968–1976.

Taylor, D. L., and Y-L Wang. 1980. Fluorescently labelled molecules as probes of the structure and function of living cells. *Nature (Lond.).* 284: 405–410.

Theurkauf, W. E., H. Baum, J. Bo, and P. C. Winsink. 1986. Tissue-specific and constitutive α-tubulin genes of *Drosophila melanogaster* code for structurally distinct proteins. *Proc. Natl. Acad. Sci. USA.* 83:8477–8481.

Tomasek, J. J., and E. D. Hay. 1984. Analysis of the role of microfilaments and microtubules in acquisition of bipolarity and elongation of fibroblasts in hydrated collagen gels. *J. Cell Biol.* 99:536–549.

Vandre, D. D., P. Kronenbusch, and G. G. Borisy. 1984. Interphase-mitosis transition: microtubule rearrangements in cultured cells and sea urchin eggs. *In* Molecular Biology of the Cytoskeleton. G. G. Borisy, D. W. Cleveland, and D. B. Murphy, editors. Cold Spring Harbor Press, Cold Spring Harbor, NY. 3–16.

Wadsworth, P., and R. D. Sloboda. 1983. Microinjection of fluorescent tubulin into dividing sea urchin cells. *J. Cell Biol.* 97:1249–1254.

Walter, M. F., and B. M. Alberts. 1984. Intermediate filaments in tissue culture cells and early embryos of *Drosophila melanogaster*. *In* Molecular Biology of Development. UCLA Symp. Mol. Cell Biol. New Series. E. H. Davidson, and R. A. Firtel, editors. Alan R. Liss, Inc. New York. 263–272.

Warn, R. M. 1986. The cytoskeleton of the early *Drosophila* embryo. *J. Cell Sci.* 5(Suppl.):311–328.

Warn, R. M., and R. Magrath. 1982. Observations by a novel method of surface changes during the syncytial blastoderm stage of the *Drosophila* embryo. *Dev. Biol.* 89:540–548.

Warn, R. M., and A. Warn. 1986. Microtubule arrays present during the syncytial and cellular blastoderm stages of the early *Drosophila* embryo. *Exp. Cell Res.* 163:201–210.

Warn, R. M., R. Magrath, and S. Webb. 1984. Distribution of F-actin during cleavage of the *Drosophila* syncytial blastoderm. *J. Cell Biol.* 98:156–162.

Warn, R. M., L. Smith, and A. Warn. 1985. Three distinct distributions of F-actin occur during the divisions of polar surface caps to produce pole cells in *Drosophila* embryos. *J. Cell Biol.* 100:1010–1015.

Weber, K., T. Bibring, and M. Osborn. 1975. Specific visualization of tubulin containing structures in tissue culture cells by immunofluorescence. Cytoplasmic microtubules, vinblastine-induced paracrystals and mitotic figures. *Exp. Cell Res.* 95:111–120.

Wehland, J., and M. C. Willingham. 1983. A rat monoclonal antibody reacting specifically with the tyrosylated form of α tubulin. II. Effects of cell movement, organisation of microtubules and intermediate filaments, and arrangement of Golgi elements. *J. Cell Biol.* 97:1476–1490.

Wehland, J., H. C. Schroder, and K. Weber. 1984. Amino acid sequence requirements in the epitope recognised by the α-tubulin-specific rat monoclonal antibody YL 1/2. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:1295–1300.

Wehland, J., M. C. Willingham, and I. V. Sandoval. 1983. A rat monoclonal antibody reacting specifically with the tyrosylated form of α tubulin. I. Biochemical characterization, effects on microtubule polymerization in vitro and microtubule polymerization and organization in vivo. *J. Cell Biol.* 97: 1467–1475.

Zalokar, M., and I. Erk. 1976. Division and migration of nuclei during early embryogenesis of *Drosophila melanogaster*. *J. Microsc. Biol. Cell.* 25: 97–106.