

Nucleus-associated Microtubules Help Determine The Division Plane of Plant Epidermal Cells: Avoidance of Four-way Junctions and the Role of Cell Geometry

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Abstract. To investigate the spatial relationship between the nucleus and the cortical division site, epidermal cells were selected in which the separation between these two areas is large. Avoiding enzyme treatment and air drying, *Datura stramonium* cells were labeled with antitubulin antibodies and the three-dimensional aspect of the cytoskeletons was reconstructed using computer-aided optical sectioning.

In vacuolated cells preparing for division, the nucleus migrates into the center of the cell, suspended by transvacuolar strands. These strands are now shown to contain continuous bundles of microtubules which bridge the nucleus to the cortex. These nucleus-radiating microtubules adopt different configurations in cells of different shape. In elongated cells with more or less parallel side walls, oblique strands radiating from the nucleus to the long side walls are presumably unstable, for they are progressively realigned into a transverse disc (the phragmosome) as broad, cortical, preprophase bands (PPBs) become tighter. The phragmosome and the PPB are both known predictors of the division plane and our observations indicate that they align simultaneously in elongated epidermal cells. These observations suggest another hypothesis: that the PPB may contain microtubules polymerized from the nuclear surface. In elongated cells, the majority of the radiating microtubules, therefore, come to anchor the nucleus in the transverse plane, consistent

with the observed tendency of such cells to divide perpendicular to the long axis.

In nonrectangular isodiametric epidermal cells, which approximate regular hexagons in section, the radial microtubular strands emanating from the nucleus tend to remain associated with the middle of each subtending cell wall. The strands are not reorganized into a single dominant transverse bar, but remain as a starlike array until mitosis. PPBs in these cells are not as tight; they may only be a sparse accumulation of microtubules, even forming along non-diametrical radii. This arrangement is consistent with the irregular division patterns observed in epidermal mosaics of isodiametric *D. stramonium* cells.

The various conformations of the radial strands can be modeled by springs held in two-dimensional hexagonal frames, and by soap bubbles in three-dimensional hexagonal frames, suggesting that the division plane may, by analogy, be selected by minimal path criteria. Such behavior offers a cytoplasmic explanation of long-standing empirically derived "rules" which state that the new cell wall tends to meet the maternal wall at right angles. The radial premitotic strands and their analogues avoid taking the longer path to the vertex of an angle where a cross wall is already present between neighboring cells. The resultant tendency to form three-rayed vertices rather than intercrossing septa helps explain the hexagonal packing of plant cells.

THE form of a plant tissue or organ depends largely on the planes in which the founding cells divide. Organized growth implies organized division planes and this reduces to the way in which the new cross wall is aligned across the dividing cell.

There are few clues concerning alignment of the division plane. A major landmark was Sinnott and Bloch's (1940) description of division in vacuolated plant cells. In preparing for division, the nucleus migrated from the cortical cytoplasm into the center of the vacuole where it was suspended

by transvacuolar, cytoplasmic strands. Gradually, most of these radiating strands coalesced into a more or less continuous transverse raft, termed the phragmosome. Because the phragmosome later formed the path taken by the centrifugally growing cytokinetic apparatus, the premitotic device clearly predicted the future division plane. There are unresolved questions concerning the phragmosome. For example, what cytoskeletal structures support it? In carrot suspension cells, the phragmosome was recently shown (Traas et al., 1987) to contain actin filaments that caged the nucleus

and anchored it to the cortex throughout division. The picture for microtubules (MTs)¹ in large vacuolated cells is less clear, probably because of the difficulty of handling such cells for microscopy. However, Bakhuizen et al. (1985) have been able to see MT segments in the phragmosome of *Nautilocalyx lynchii* epidermal cells by EM. The MTs in the phragmosome were suggested to be more stable than MTs radiating from the nucleus in other directions, for the latter class did not persist. In this paper, we report that MTs radiating from the nucleus during premitosis are not fragmentary, but form a continuous network. The realignment of the radial MTs into the transverse phragmosomal baffle, during the premitotic period, is described.

Another, better-known predictor of the division plane is the preprophase band (PPB) of MTs (Pickett-Heaps and Northcote, 1966). This cortical ring disappears before metaphase yet it is known to predict successfully a variety of complex division planes (see Gunning, 1982). Recently, the PPB has been found to contain actin filaments as well as MTs (Palevitz, 1987; Traas et al., 1987; Kakimoto and Shibaoka, 1987; Lloyd and Traas, 1988). Although actin filaments, like the MTs, seem to disappear from the PPB by metaphase, the nucleus-radiating actin filaments in the phragmosomal plane remain. In the present paper, we examine the relationship between the nucleus-radiating MTs and the cortical MTs of the PPB; we discuss the coincidence between the narrowing of the cortical band and the gradual realignment of the majority of the nucleus-radiating MTs into the phragmosomal plane bounded by the PPB.

Over and above the cytoskeletal mechanics of the division plane formation is the larger issue of its alignment, for this has a major impact on tissue organization. That cells tend to divide in a particular plane was long ago embodied in empirical rules (Hofmeister, 1863; Sachs, 1878; Errera, 1888) that attempted to account for the way in which the new cell plate tended to contact the maternal (long) wall at right angles. Counter examples can be produced (see Gunning, 1982) but the "rules" are at least an attempt to explain basic features of cell packing and geometry. A characteristic of most unwounded plant tissues is that cells in adjacent files appear to be staggered like bonded bricks in a wall (Sinnott and Bloch, 1941; Thompson, 1942). In such patterns, only three vertices meet at any one point. This means that there must be an avoidance mechanism whereby new cross walls tend not to insert upon the maternal wall opposite the point where a neighboring cross wall is already attached. The premitotic microtubular strands that radiate from the nucleus exhibit a similar avoidance of existing three-way junctions. This behavior can be mimicked by analogue models and offers a cytoskeletal reinterpretation of empirical rules for cell packing.

Materials and Methods

Plants of *Datura stramonium* L. were grown in a glasshouse as described by Flanders et al. (1989). Split sections of internode, ~1 cm long, were fixed for 4 h, initially aided by vacuum infiltration, in freshly prepared 4% (wt/vol) formaldehyde in extraction buffer (50 mM Pipes, 5 mM EGTA, 5 mM MgSO₄, 1% (vol/vol) DMSO, 0.1% (vol/vol) NP-40, pH 6.9). After several rinses, strips of outer tissue were removed with a flexible razor blade. The strips were then scored with a razor blade from the internal face

1. *Abbreviations used in this paper:* MT, microtubule; PPB, preprophase band.

to the outer epidermal wall. Only cut cells stain, but this method avoids the distortions of air drying and separation by enzymes. The strips were then gently placed into chamber slides and stained with YOL 1/34 (Kilmartin et al., 1982) anti-tubulin and second antibody, as described fully by Flanders et al., (1989). Both cortical and endoplasmic staining patterns were specific to anti-tubulin and were not observed when primary antibody was omitted.

Data Collecting and Image Processing

The equipment and methods used for three-dimensional image data collection have been described in detail elsewhere (Traas et al., 1987; Lloyd et al., 1987; Rawlins and Shaw, 1988). In brief, the epifluorescence image from a Universal microscope (Carl Zeiss, Ltd., Welwyn Garden City, England) is relayed to an ISIT video camera and the video image is digitized by a framestore interfaced to a VAX 11/750 computer. The fine focus of the microscope is under computer control via a microstepping motor drive.

Optical sections were collected at regularly spaced focus levels (in this study at 1 or 2 μ m) through the specimen. Video frame averaging (generally over 256 frames) was used to reduce noise in the video images.

Each optical section consisted of 512 \times 512 pixels, with an interpixel spacing of 0.14 μ m. At the end of the collection of the data stack, a background, out-of-focus image was recorded and was subtracted from each image in the data stack before further processing.

Removal of out-of-focus information (deblurring) was carried out using the simple nearest neighbor algorithm described by Castleman (1979) and Agard et al. (1989). To visualize the MTs from all sections simultaneously (i.e., to reconstruct the whole-cell MT array), series of related projections of the three-dimensional reconstruction were calculated in small angular increments between + and - 24° using the program described by Agard et al. (1989). To give the effect of rotation of the reconstructed array, the projections were displayed in rapid sequence. In addition, stereo pairs were produced by displaying two projections side by side on the monitor. Finally, the stereo pairs themselves could be "rotated" on the screen. The images shown in this paper were obtained by a videofilm recorder (4500; Ramtek Corp., Santa Clara, CA) using Panatomic-X film (Eastman Kodak Co., Rochester, NY).

Analogue Models

A pin-jointed, hexagonal prism was constructed of 1.5-mm-diam stainless steel rod. The upper and lower hexagonal frames (with 3-cm sides) were separated by 1-cm steel tubes. Rafts of bubbles, formed from a detergent/water solution stabilized by 10% (vol/vol) glycerol, were then picked up in the prismatic frame.

A two-dimensional hexagonal frame was constructed of six pin-jointed pieces of 5-cm long stainless steel tubing. A disk was suspended in the center of the frame by six steel springs, each of which connected with a Teflon collar capable of sliding along each edge, between pin joints.

Results

In a previous paper, the epidermal cells of *D. stramonium* were described as falling into two loose categories: elongated cells with more or less orthogonal facets, and isodiametric cells with no defined long axis and a predominance of non-orthogonal facets (Flanders et al., 1989). These cells were obtained from epidermis of the internode below the stem's bifurcation; that is, mature tissue with residual mitotic activity. In such large vacuolated cells an early indication of ensuing mitosis is the migration of the nucleus from the cortex into the center of the vacuole where the nucleus is suspended by transvacuolar strands (Sinnott and Bloch, 1940, 1941; Venverloo et al., 1980). Later, generally accepted indicators of preprophase (i.e., PPB of cortical MTs, perinuclear MT fluorescence; Wick and Duniec, 1983) are seen. Events associated with the earlier migration of the nucleus, preparatory to division, are referred to here as "premitotic."

Fig. 1 presents examples of PPBs in the regular, elongated cells with more or less parallel opposite walls. In *a*, a transverse PPB can be seen forming among other cortical MTs. At a deeper level of focus (*b*) the immunofluorescent halo of

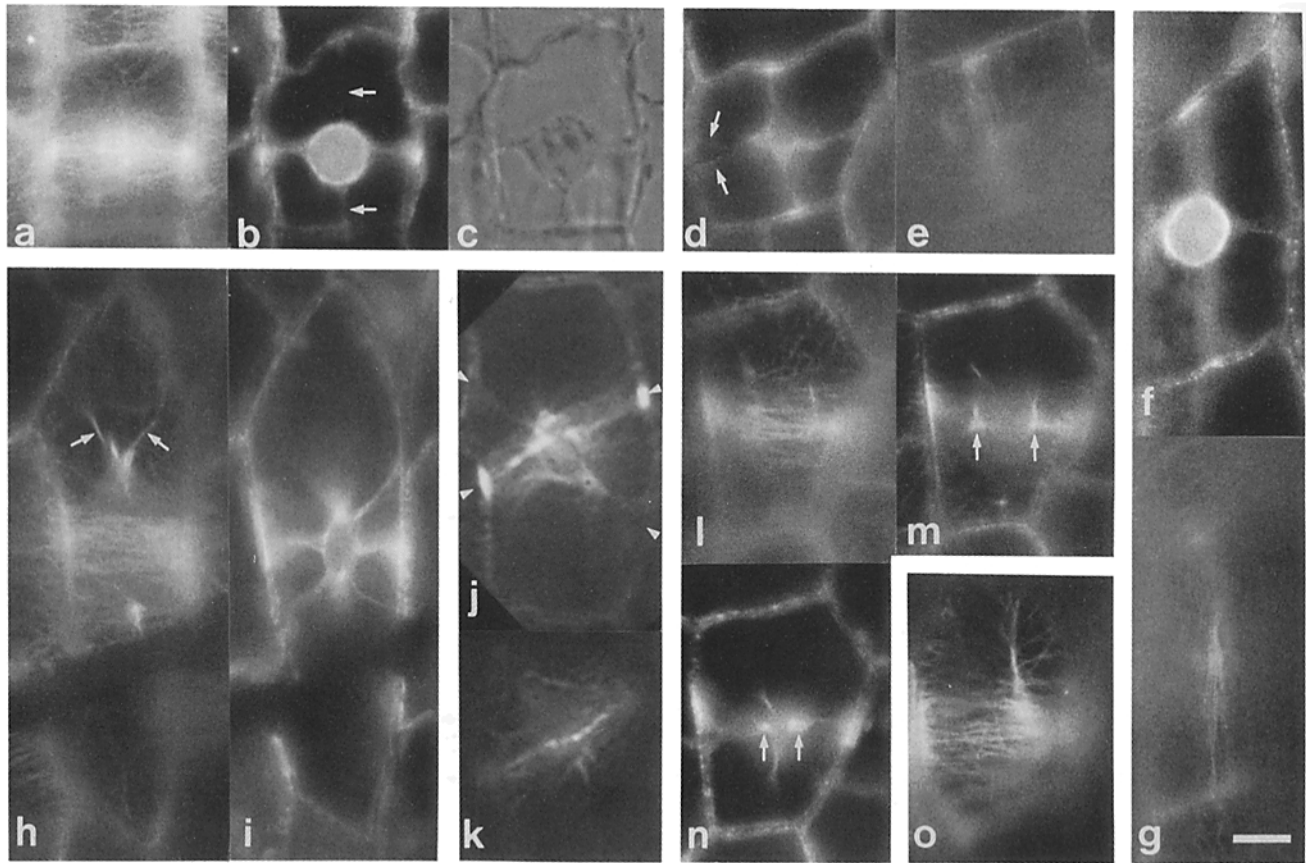


Figure 1. Antitubulin labeling of elongated premitotic *Datura stramonium* epidermal cells. The long axis of the stem lies up and down the page. (a) Tight PPB forming at the outer epidermal wall. (b) The same cell, focused at the nucleus, shows the PPB end-on, connected to the cortex by an accumulation of transverse strands. Perpendicular to this, slender polar plasmic (arrows) can be seen. (c) Nomarski image of b. (d) Similar to a–c except with a longitudinal PPB. Note the polar plasmic strands bifurcate (arrows) either side of an existing three-way junction. (e) Surface view of longitudinal PPB. (f) Another longitudinal division plane with an elongated cell; note the perinuclear fluorescence. (g) Surface view of longitudinal PPB. (h) Surface view of broad PPB forming in elongated cell with gabled end walls. The polar plasmic strands (arrows) bifurcate outside the PPB zone, and are directed toward the middle of the end walls. (i) Focused at the nucleus, radial microtubular strands lie within the PPB zone. (j) Reconstruction of 21 (z axis spacing = 1 μm) sections taken from the center of the cell, showing an X-shaped accumulation of nucleus-associated strands (arrowheads). The strands to the lower edge of the cell straddle an existing three-way junction. The PPB, as seen by the intense fluorescence down the anticlinal walls, is diagonal and has only formed along one limb of the cross. (k) Single section at the surface of the cell shown in j. Radial strands in the z axis can be seen end-on as the bright dots within the plane of the diagonal PPB. (l) PPB forming within slightly irregular, but still-elongated cell. (m) The same cell, focused just beneath the surface showing two strands (arrows) of endoplasmic MTs. (n) These same strands (arrows) can be followed to the nucleus. The PPB can also be followed, end-on, as accumulated fluorescence down the side walls. (o) In another cell, focused at the surface, a thick strand of MTs passes perpendicularly away from the PPB. Other cortical MTs can clearly be seen radiating from this strand. Bar, 10 μm .

the preprophase nucleus is visible. The nucleus is connected to the cortical PPB by MTs that occupy the transvacuolar division plane and are therefore visible at each level of focus, constituting the phragmosome. Other MTs (arrows) are perpendicular to those within the division plane. These correspond to the polar strands described by Sinnott and Bloch (1940) and to the nonphragmosomal strands stained with rhodamine phalloidin by Lloyd and Traas (1988) in carrot cells. The polar strands, unlike the phragmosomal MTs, cannot define a transvacuolar plane for they are thin filaments. The Nomarski image (c) indicates that most of the transvacuolar cytoplasm is transversely arranged. Sinnott and Bloch (1940) referred to the phragmosomal and polar strands as forming a Maltese cross. However, note that although the Maltese cross adequately describes the strands in two dimen-

sions, it ignores strands in the third dimension. The phragmosome (the transverse bar of the Maltese cross) is more like a bicycle wheel with the nucleus at the hub, the PPB at the rim, and the transvacuolar strands as the spokes. The polar strands, like the axle of the wheel, are perpendicular to this plane. This configuration, rotated through 90° in the plane of the page, also applies when cells divide parallel to the stem's long axis (d and e). The difference between b and d is that the polar strands in d bifurcate and straddle (arrows), rather than join, an existing junction with a neighboring cell.

When elongated cells divide parallel to the cell's long axis the Maltese cross configuration also applies (f and g).

These are quite well-developed PPBs, but in the long cell shown in (h and i) the PPB is broader and presumably, therefore, in the act of forming. The endoplasmic MTs radiating

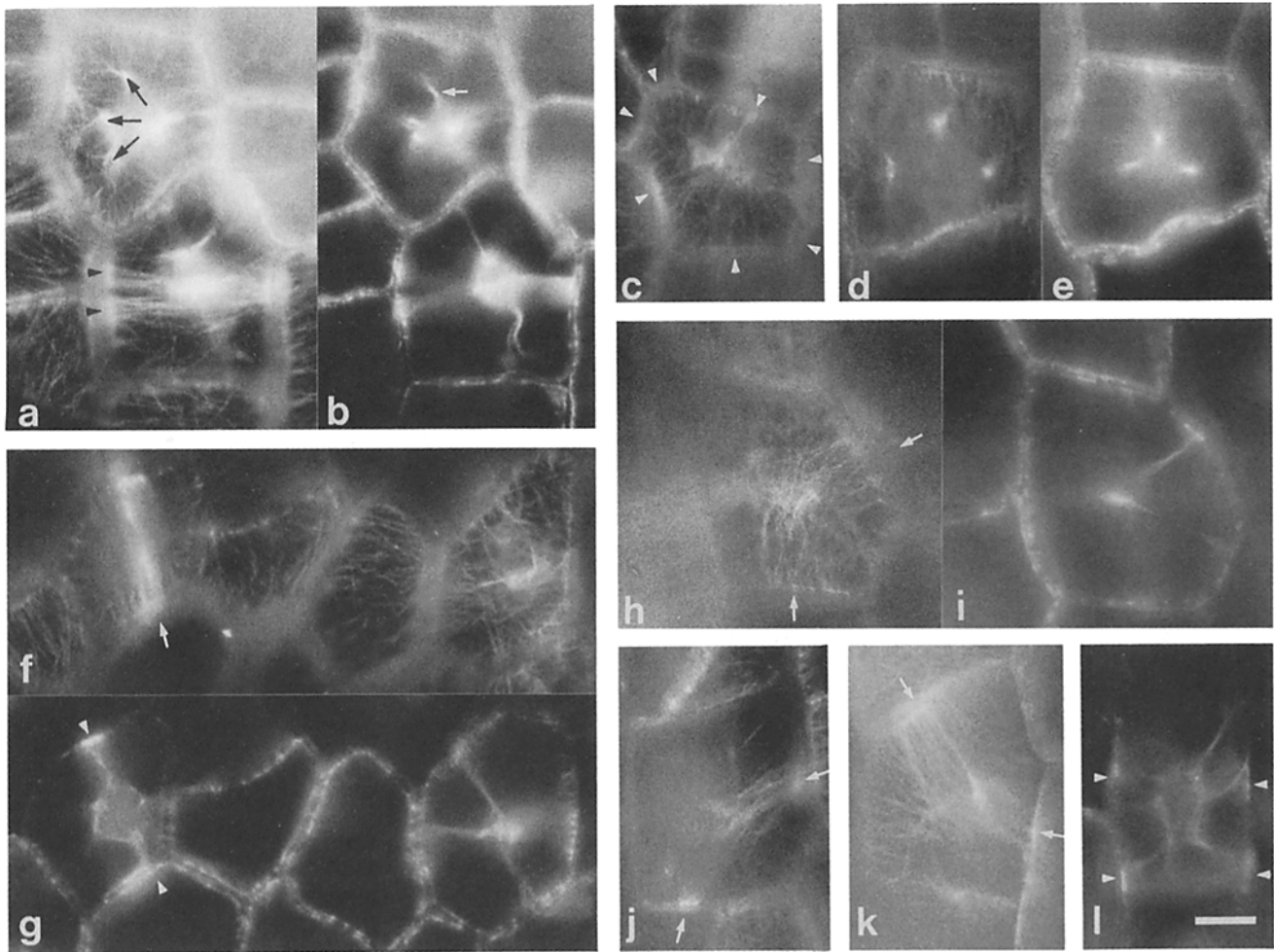


Figure 2. Antitubulin labeling of isodiametric, premitotic epidermal cells. (*a* and *b*) In the upper cell in *a*, cortical MTs radiate from several points of intense fluorescence (e.g., *black arrows*). These can be traced in *b* (e.g., *white arrow*) to strands radiating from the nucleus. In the lower cell (*a*), the PPB is regular to the right, but to the left (*arrowheads*) bifurcates either side of a three-way junction. (*c*) Outer epidermal wall of an isodiametric wall. MTs splay out from a central accumulation, meeting all side walls more or less perpendicularly (*arrowheads*). (*d* and *e*) Three intense spots of fluorescence, among the MTs on the outer epidermal wall (*d*), can be traced down to three strands converging upon the nucleus (*e*). (*f* and *g*) The cell to the left has a tight PPB (*arrow*) forming across its outer wall between two parallel opposite walls. By focusing down (*g*) radial strands (*arrowheads*) can be seen anchoring the nucleus within the division plane. The cell to the right in *f* has a diffuse PPB forming between nonparallel anticlinal edges. The underlying radiating strands (*g*) form an X. (*h* and *i*) Example of a diffuse, broad PPB forming between nondiametrically opposed walls (*h*, *arrows*). The band lies on two radii, which meet above the nucleus. By focusing down through this point, a column of MTs can be seen (*i*) to connect with the central nucleus. (*j* and *k*) Two further, somewhat tighter, angled PPBs forming on the outer epidermal wall between nonparallel cell edges. (*l*) The central portion of a much-elongated subepidermal cell showing double, transverse PPBs forming between the arrowheads. At this level of focus, endoplasmic MT-containing strands can be seen running from the nucleus to the bands. Bar, 10 μm .

between nucleus and cortex (*i*) do not, at this stage, define the transverse plane; they make contact with the cortex within the greater zone bracketed by the broad PPB. Note the polar strands (*h*, *arrow*) which bifurcate as they are directed towards the angled end walls.

j represents several recombined focal sections from the middle of an elongated cell. Here, the radiating microtubular strands constitute, in section, a flattened X (*arrowheads*). To the left, the bifurcated strands avoid contact with the junction formed by three existing vertices. The PPB, however, is well formed and tight but has clearly only developed along one limb of the X. The inset (*k*) presents a single section from the cortex of this long cell. Strands radiating from the nucleus to the outer epidermal wall (in the plane of the page) can be seen end-on as dots, along the diagonal line of the

canted PPB. This alignment of the radiating strands within the plane of a mature (tight) PPB is to be contrasted with the still-radiating conformation where the forming PPB is broad (*h* and *i*).

The realignment of the radiating, nucleus-associated MTs, during PPB formation is further illustrated in *l*, *m*, and *n*. The broad PPB is shown at the cortex in *l*, but just below the PPB (*m*) two columns of MTs (*arrows*) can be traced to run between nucleus and cortex in the *z* axis. These focus down to two bright spots (*n*, *arrows*) at the nuclear surface, where polar strands also pass away perpendicularly. In the cell shown in *o*, a bundle of MTs runs perpendicular to a broad PPB. Smaller groups of MTs radiate from this bundle, outside the PPB zone, and diverge upon the cortex.

In summary, MTs radiate between the nucleus and cortex

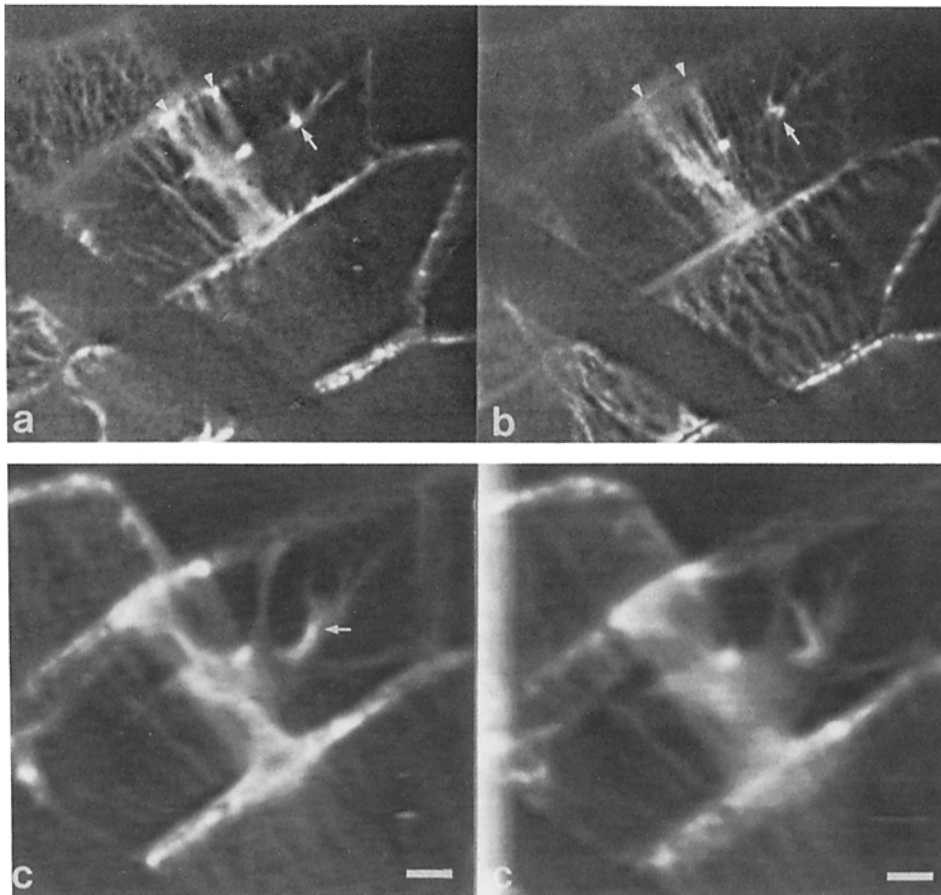


Figure 3. Computer reconstructions of a PPB forming in an elongated epidermal cell. (a and b) Computer deblurred sections from just below (a) and at the cortex (b). A PPB is forming among other MTs, straddling (arrowheads) an existing three-way junction with a neighboring cell. An endoplasmic strand outside the PPB zone (arrows) terminates at the cortex where MTs radiate away from the band. a and b provide cortical detail not seen in the following stereo projection in which many, rather than single, sections are displayed. (c) Stereo projections of 24 recombinated sections ($z = 2 \mu\text{m}$) from nucleus to cortex. The nucleus is suspended centrally by strands within the PPB zone. Another thick strand radiates from the nucleus perpendicular to the band. At the arrow, this strand subdivides, at a point corresponding with the arrow in a, before splaying out upon the cortex. Cortical MTs to the left of this junction (see a and b) are parallel with the forming PPB, whereas MTs to the distal end of the cell (right) radiate in a nonparallel manner. Bars, $5 \mu\text{m}$.

during PPB formation. Where the cortical band is tight and well developed, most of the MTs radiating from the nucleus occur in the phragmosomal plane and with minority polar strands perpendicular to this. Where the band is broad and presumably still forming, endoplasmic MT-containing cytoplasmic strands diverge from this conformation; some are still within the greater cortical zone defined by the broad band, while others tend to run away perpendicularly, towards the end walls. Therefore, regular PPBs form between parallel opposite side walls. The polar plasmic strands are capable of splitting towards angled end walls, or avoiding existing three-way junctions.

Premitotic Arrangement of Radiating Strands in Isodiametric, Nonorthogonal Cells

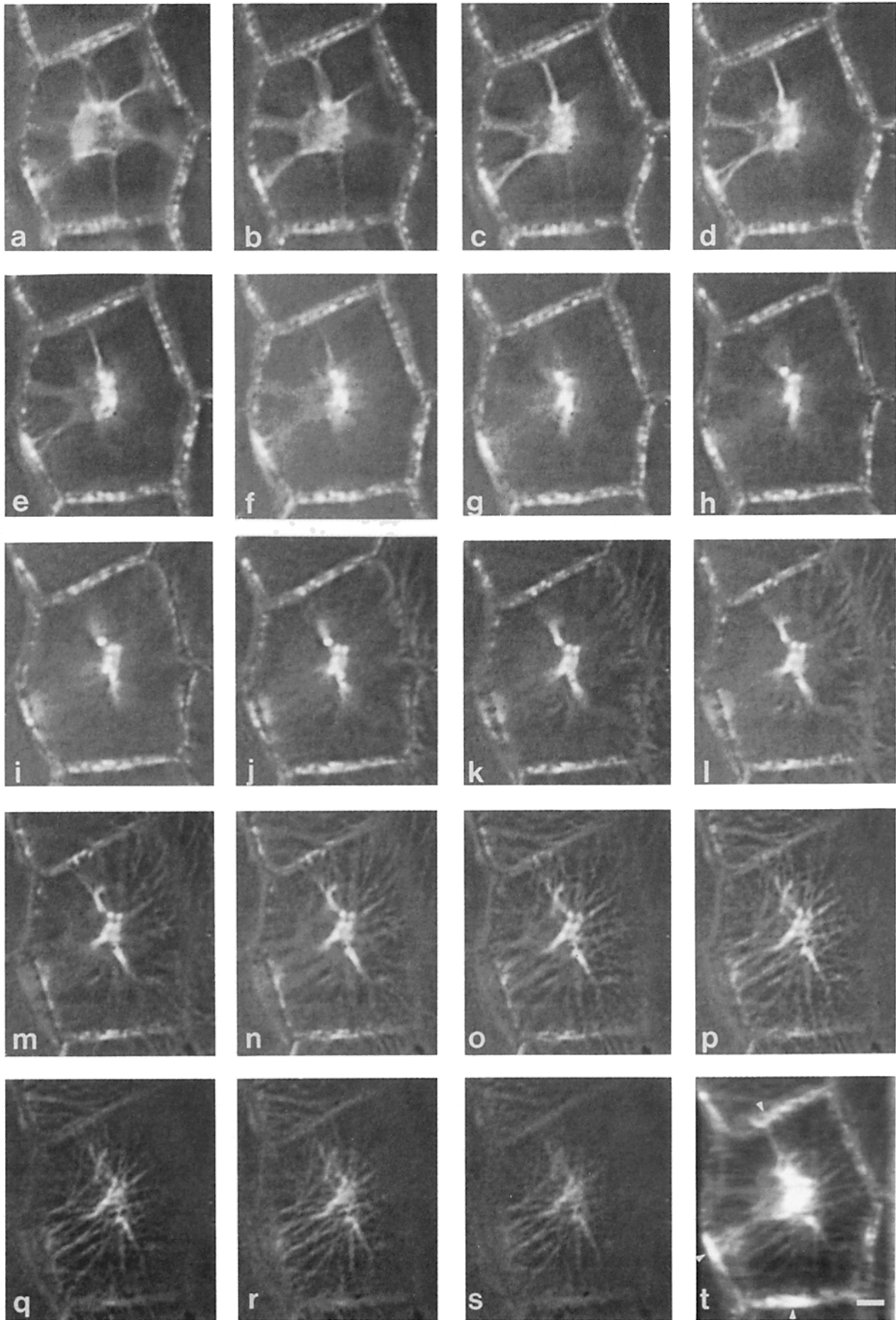
Fig. 2 a shows two cells, each with a central nucleus, hence preparing for division. In the lower cell a PPB is forming across the rectangular bottom half of the cell. To the right the band is tight, but to the left it bifurcates (arrowheads), avoiding the existing three-way junction. Focusing upon the nucleus (b) it can be seen that the endoplasmic strands form, in this focal plane, a regular Maltese cross (but with the left arm split, slightly out of focus). However, in the upper of the two cells in a there are several centers of bright antitubulin fluorescence from which MTs are radiating upon the cortex. From each of these centers, the cortical MTs run perpendicu-

larly to the nearest cell edge. These centers can be traced by through-focusing (b) as thick strands (e.g., arrow) that converge upon the nucleus.

A similar arrangement can be discerned more clearly in c. There is a central column of MTs that passes between the nucleus and the middle of the outer epidermal wall; MTs radiate from the center of this wall to contact each anticlinal cell edge (arrowheads) perpendicularly. Again, in d three centers of fluorescence can be observed just beneath the cortex. Upon focusing down from this outer epidermal wall, the three structures can be traced as three endoplasmic microtubular columns (e) that converge upon the central premitotic nucleus.

PPB Formation in Isodiametric, Nonorthogonal Cells

In these less regular isodiametric cells, PPBs can still form between two more or less parallel opposite walls (e.g., 2 a). This is also seen in the cell to the left in f. However, in the rhomboidal cell to the right in f, the broad band is less well organized. It is difficult to discern the pair of opposite walls between which the band will form. The endoplasmic MT strands form an X in contrast to the dominant phragmosomal strands formed in the left-hand cell (g). It is possible that the right-hand cell represents a transitional stage but after examining a large number of nonorthogonal isodiametric cells we have been unable to find a tight PPB that circumnavigates



such cells. Intriguingly, such cells can form broad PPBs between nondiametrically opposite anticlinal walls: that is, the bands can be angled. A band of MTs is in a two and six o'clock hour-hand configuration (*h* and *i*, arrows). In *j*, where the band is better delineated, the MTs are in a three and seven o'clock arrangement. In *k* the cortical premitotic MTs are in an 11 and 4 o'clock configuration. In each of these cases the angle in the band is directly above the nucleus to which it is connected by MTs. Such connections were further studied by computer-aided optical tomography (below). Curved division planes, as anticipated by such angled bands, are actually formed in this tissue as described by Flanders et al. (1989).

Finally, in *l*, a long subepidermal cell is shown which contains a double PPB (arrowheads). It can be seen that MT-containing strands radiate from the nucleus to each band.

Computer-aided Three-dimensional Reconstruction of PPB Formation

Elongated Cells. To study the connection between the nucleus and the PPB, cells were optically sectioned and out-of-focus fluorescence was removed by computer methods. Fig. 3 *a* presents a subsurface section of an elongated cell in the process of forming a PPB. The process appears to be well advanced as judged by the central accumulation of MTs. It is noteworthy that the band along the upper cell edge splits (arrowheads) either side of a three-way junction formed by a perpendicular end wall between neighbors in the adjacent file. In this plane of focus (*a*) a bright dot of fluorescence can be seen within the band itself, as well as a further dot (arrow) and cables to the right along the central longitudinal axis. In the following section, at the cortex itself (*b*), the arrowed dot to the right can be seen to be the center for MTs radiating upon the cortex, away from the band.

Fig. 3 *c* was prepared by recombining the focal series from the outer half of a cell and projecting this as a stereo pair in order to demonstrate the relationship between nucleus and cortex. The effect is of looking at the outer epidermal wall (seen in *b*) from the inside of the cell. By rotating the recombined sections to the right, the three bundles of MTs (*a*, arrow) can now be seen to be part of a larger bundle that connects with the nucleus. The nucleus itself is connected within the division plane, to two opposite anticlinal walls, by other bundles of MTs.

Nonorthogonal Isodiametric Cells. From the light micrographs (e.g., Fig. 2) and from through-focusing, PPBs do not appear to be as well developed in isodiametric cells as in elongated cells.

Fig. 4 represents computer deblurred 1- μ m sections, from nucleus to outer epidermal wall, of a premitotic cell in which the nucleus has assumed a central position and cortical MTs

are beginning to bunch up. First, the series shows (*a-e*) that MT-containing strands radiate from the nucleus to the center of each of the six subtending facets or cell edges in that plane. The outer epidermal wall is unlike any of these six anticlinal walls because it has no neighbors. This unfaceted outer wall is domed, as signified by the fact that several sections (e.g., *l-r*) are required to map MTs running from its edges to its center. Sections *d-j* trace a set of antitubulin-stained fluorescent bundles, from the central nucleus up towards the outer epidermal wall. These central bundles splay out in a cross-like fashion towards the outer epidermal wall (*k-n*). Upon contacting the cortex this central column of endoplasmic MTs radiates upon the surface, tending to meet each cell edge more or less perpendicularly. It is important to appreciate that these cortical MTs radiate from the center of the cell face; at this stage there is no bunching up of MTs on the outer epidermal wall as might be expected if side-to-side accumulation of cortical MTs were the mechanism for drawing the subtending endoplasmic MTs into a central column. Although no PPB can be seen on the outer epidermal wall, the projected series of sections presented in *t* reveals accumulation of MTs down three of the anticlinal walls.

In contrast, Fig. 5 *a* is a stereo pair of an isodiametric cell in which a PPB is forming on the outer epidermal wall. The inner epidermal wall has been edited out in order to show a nucleus suspended within an open box composed of six anticlinal walls and the periclinal outer epidermal wall. The nucleus can be seen, suspended centrally by MT-containing strands. MTs present a burst of fluorescence as they are viewed, end-on, along the anticlinal wall to the upper right (arrow). These then pass obliquely across the outer epidermal wall to the opposite arrow at the lower left. However, this oblique band bifurcates behind the nucleus and a branch emerges on the tilted anticlinal wall to the left (arrowhead). This bifurcation of the band can be seen by rotating the reconstituted sections on screen (not presented). To demonstrate the point in static images, most of the central sections including the nucleus have been edited out in *b*. In this stereo pair, the three dots are vestiges of three endoplasmic MT strands that pass from the nucleus to the outer epidermal wall. The oblique PPB can be seen (as in *a*) passing from top right to bottom left except that now, without the nucleus, the endoplasmic strands can be viewed as the pivotal point where PPB MTs bifurcate. They bifurcate towards the anticlinal wall to the left, along which its own PPB fluorescence can be viewed end-on (arrowhead).

Discussion

Two main points emerge from these results. One is that in premitotic cells, bundles of MTs unambiguously bridge the

Figure 4. Deblurred but unprojected serial sections, from the nucleus to the outer epidermal wall, of an isodiametric cell. In *a-e*, radial microtubular strands pass from the nucleus to the middle of each anticlinal wall. In *e-i*, a column of MT containing strands lies along the z axis from the nucleus toward the middle of the outer epidermal wall. These then splay out in the shape of a cross (e.g., *n*). In subsequent sections up to the cortex, MTs are seen to radiate out from the center of the wall, running perpendicular to the cell edges. In *t*, the foregoing sections ($z = 1 \mu\text{m}$) are combined. Arrows indicate that incipient accumulations of MTs are forming down three anticlinal walls whereas no PPB formation is at this stage visible on the outer epidermal wall. Therefore, in this premitotic isodiametric cell a stellate array of cortical MTs focuses upon central columns of endoplasmic MTs that bridge nucleus to cortex. Such columns also radiate between nucleus and the mid-tangential anticlinal walls. Bar, 5 μm .

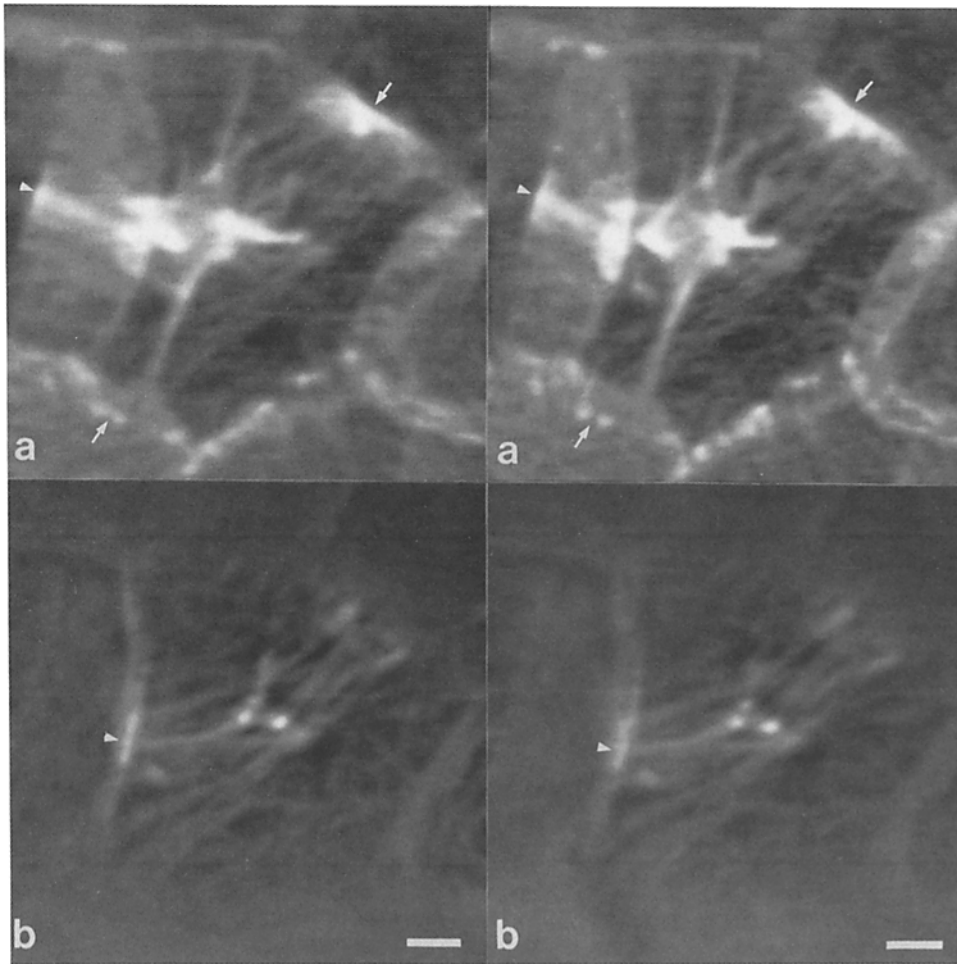


Figure 5. Stereo projections of angled PPB formation in an isodiametric cell. In the outer portion of the cell displayed here, the nucleus is suspended on MT containing strands (*a*). On the outer epidermal wall, a broad PPB appears to be forming obliquely, between the arrows. Behind the nucleus, however, the band bifurcates, emerging as a strong PPB on the anticlinal wall to the left (*arrowhead*). In *b*, only a few cortical sections (in the same orientation as *a*) are projected. These show that cortical MTs run from the junction between radiating endoplasmic strands and cortex, to the PPB which is seen end-on along the anticlinal wall (*arrowhead*). In *a*, therefore, the PPB on the upper cell edge, and the PPB to the left, are connected across the outer epidermal wall by nondiametrical radii which pivot around that junction with the transvacuolar MT strands. 25 sections ($z = 2 \mu\text{m}$). Bars, $5 \mu\text{m}$.

large gap between nucleus and cortex in highly vacuolated cells. This has implications both for nuclear anchorage and for PPB formation. The second is that different configurations of these nucleus-associated strands occur in orthogonal and nonorthogonal cells, in line with observed differences in division pattern. This throws light upon the larger morphogenetic issues of division plane alignment and cell geometry.

MTs Connect the Nucleus to the Cortex during Premitosis

The entire process and mechanism of division plane alignment is more exposed within large vacuolated cells than in dense cytoplasmic cells. In vacuolated cells, the nucleus migrates from the cortex into the center of the cell where it is suspended by transvacuolar strands in preparation for division (Sinnott and Bloch, 1940, 1941; Venverloo et al., 1980). Sinnott and Bloch (1940) observed that the nucleus-associated transvacuolar strands gradually realigned into a more or less continuous baffle across the cell. This was termed the phragmosome. Other cytoplasmic strands, not drawn into the phragmosomal plane, ran perpendicularly from the nucleus to the end walls but seemed to disappear around metaphase. The attitude of the spindle may change during division and is not a good predictor of the division plane (see also Gunning, 1982). The phragmosome, on the other hand, remained

in a set plane throughout mitosis. The cytokinetic apparatus developed within it and hence phragmosome formation was known to anticipate the future division plane a quarter of a century before the cortical division site was shown by Pickett-Heaps and Northcote (1966) to be predicted by the PPB. This band has subsequently been found to anticipate even curved division planes in a broad range of cell types (see Gunning, 1982). A major problem, in terms of cytoskeletal mechanism, has been the detection of elements that anchor the dividing nucleus in the center of the cell and connect it with the cortical cytoplasm marked by the PPB MTs.

In an EM study of developing stomatal complexes in wheat, Pickett-Heaps (1969) observed MTs sectioned between the nucleus and the PPB. It was inferred that these might be spindle MTs in the process of moving intact from the PPB. Later, Bakhuizen et al. (1985) also saw in the EM that the phragmosome of highly vacuolated *Nautilocalyx* cells contained MTs during the transition from interphase to mitosis. They noted that MTs radiating from the nucleus in the division plane were apparently stabilized because MTs radiating in other directions did not persist. Using immunofluorescence, Wick and Duniec (1983) observed MT fluorescence between nucleus and PPB. In the densely cytoplasmic *Allium* meristematic cells it is difficult to discern individual MTs in the short gap between nucleus and cortex although such an image is presented pole-end on in Tiwari et

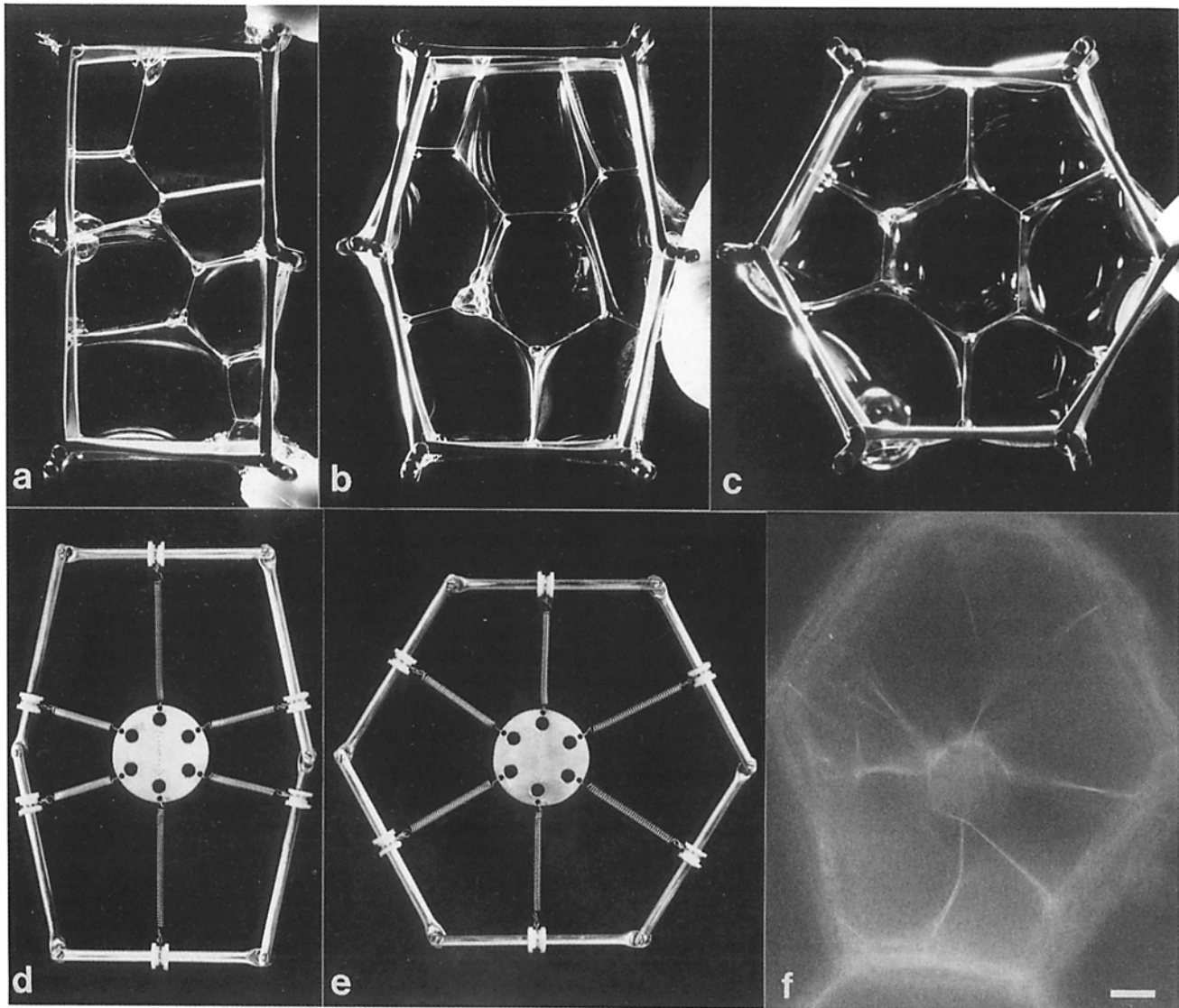


Figure 6. Analogue models demonstrating avoidance of four-way junctions. In *a* the pin-jointed frame is formed into a rectangular hexagon, ensuring that there are no angles formed down the long sides. By holding bubbles in this frame, it often occurs that bubble walls meet at the central pin joints along the long axis. This is unusual, because plant cell walls tend, if only locally, to meet at 120° (Thompson, 1942), but demonstrates that there is no mechanical reason for the walls to avoid those joints while the frame is in this configuration. However, when even slight angles are introduced between the adjacent facets of the long side walls of the frame (*b*), the bubble walls avoid making contact with the central pin joints, as they do with all other angles in the frame. The principle is best demonstrated when the frame is formed into a regular hexagon (*c*). The bubble walls, on surface tension principles, tend to meet each mid-edge since this radius is shorter than the larger radius of the circum circle required to pass through the vertices. This principle is also demonstrated when springs on Teflon collars are set within a jointed frame. In the elongated frame, with angled facets along the long side wall (*d*), the springs do not meet at the pin joints. Note the springs under tension, passing to the end walls; these resemble the polar strands that run perpendicularly from the transverse phragmosome. When the regular hexagon is formed, the springs (*e*), as for bubble walls (*c*), follow the shorter path to each mid-edge rather than the longer path to the vertices. Compare this configuration with the paths taken by transvacuolar, MT-containing strands in the regular hexagonal epidermal cells presented in Fig. 4 *b*. In Fig. 6 *f* an isodiametric epidermal cell from the white area of a variegated *Tradescantia* leaf (which, without pigmentation, can be easily stained with rhodamine phalloidin) demonstrates the presence of F-actin in radial strands. Bar, 10 μm .

al. (1984) (their Fig. 2 *b*). Mineyuki et al. (1989) have also recently observed MTs by immunofluorescence between nucleus and PPB in *Allium* guard mother cells.

In the present study, in which larger vacuolated epidermal cells are used, bundles of MTs can unequivocally be seen to radiate from the nucleus, both within and without the division plane marked by the cortical PPB. Together with the

previous evidence, this now establishes that MTs are not simply present in fragmentary form but constitute a continuous system which anchors the nucleus to the cortex. The anchoring role is not, however, likely to be exclusive to MTs, because they disappear from the cytoplasm around metaphase. In previous work, we found that actin filaments anchor the nucleus to the cortical division site throughout division

(Traas et al., 1987) and continue to connect the outgrowing phragmoplast to the former PPB site (Lloyd and Traas, 1988).

The conspicuous association of MTs with the premitotic nucleus contrasts with the apparent lack of microtubular association between nucleus and the mature cortical interphase array. This invites the question of where the nucleus-associated MTs originate at the onset of mitosis. There are two extreme (but by no means all) possibilities (see Doonan et al., 1987). One is that the MTs arise from newly activated cortical MT nucleation sites (or by a massive reorganization of preexisting cortical MTs). The other is that they arise from MT nucleation sites around the nucleus. We favor the latter possibility, because it is logistically easier to have MTs originating from a central organelle and radiating to several cell walls than vice versa. Because these transvacuolar MTs are continuous with the PPB, the possibility must be considered that the PPB contains MTs newly nucleated from the nuclear surface.

Cytoskeletal Involvement in PPB and Phragmosome Formation

Wick and Duniec (1983) have characterized broad and double PPBs as early, and tight PPBs as mature. The band, therefore, narrows during its formation and it is important to see this as background to the behavior of the nucleus-associated MTs because in *D. stramonium*, these two elements appear to reorganize hand-in-hand.

Both in isodiametric epidermal cells and in elongated cells with broad PPBs, microtubular strands radiate from the nucleus in many directions. In elongated cells with broad PPBs the radiating strands contact the cortex at various angles within the broad boundary of the forming PPB, while others, outside the band, tend to run perpendicularly to distant locations on the side and end walls. In the case of tight, presumably mature, PPBs, the oblique strands have disappeared. Instead, most of the radiating strands are, at this stage, within a defined plane tightly marked by the PPB. These are the phragmosomal MTs. Other microtubular strands run away from the nucleus, perpendicular to the phragmosomal baffle. These perpendicular strands are the polar strands described by Sinnott and Bloch (1940). The division plane (the phragmosome) is, therefore, not immediately established by an unerring outgrowth of MTs from the nucleus but seems to be arrived at gradually by a process in which oblique strands hunt the transverse axis.

In their EM study of the phragmosome in epidermal cells of *Nautilocalyx*, Venverloo et al. (1980) observed that a band of MTs marked the perimeter of the ring within which the phragmosomal strands became gathered. It could not be decided which was the first predictor of the division plane: phragmosome or PPB. From the present study it would appear that the cortical band and transvacuolar strands form a continuum such that formation of a tight PPB and formation of the planar, transvacuolar phragmosome are one and the same process: as the band condenses, the nucleus-radiating microtubular strands gather in the phragmosomal plane.

If the phragmosome is formed in part by a realignment of the radiating strands, this could account for the poor PPB formation in nonorthogonal, isodiametric cells. In these cells, unlike the elongated cells, the premitotic strands radiate to the mid-edge of the subtending cell facets and this

starlike array persists until mitosis. There appears to be no major realignment into a dominant transverse baffle of cytoplasmic strands. In such cells, the PPBs are correspondingly weak. They are not as tight as in orthogonal cells with parallel long edges and the PPBs are sometimes angled like the hands of a clock. This agrees with the variety of nontransverse and nondiametrical division planes formed by mosaic-like regions of epidermis (see Flanders et al., 1989).

The broad and angled PPBs formed in such cells appear to be a concomitant of the nonorthogonal condition rather than the isodiametric state per se. Isodiametric but orthogonal cells (e.g., Fig. 1, *b* and *d*) do form regular, tight PPBs. The irregularity displayed by some of the cells in Fig. 2 seems to be associated with PPBs attempting to form between nonparallel cell walls. In a previous paper (Flanders et al., 1989) it was shown for elongated epidermal cells that MTs passed from one cell face to another, until the transverse axis was circumnavigated. By contrast, isodiametric cells with nonorthogonal facets present a wrapping problem such that one of the facets (usually the outer epidermal) often contained a criss-cross pattern caused by MTs of divergent orientation spilling onto a common wall from angled neighboring walls. The geometrical problems of circumnavigating a nonorthogonal interphase cell with long linear elements appears to be recapitulated at preprophase, resulting in the formation of angled PPBs.

According to our observations on elongated cells, the "bunching up" of the cortical PPB and the realignment of the radial MT strands into the phragmosome, occur concomitantly. Further work is needed to decide whether narrowing of the band draws the radiating, nucleus-associated MTs into the phragmosome or whether it is realignment of the radiating MTs that helps tighten the PPB. However, several observations already indicate that positioning of the PPB can be more readily understood in terms of the underlying MTs that radiate from the nucleus than by bunching up of the PPB. For example, two sets of strands sometimes straddle a preexisting three-way junction, thus forming a split phragmosome (e.g., Figs. 1 *d*, 2, *a* and *b*, and 3 *a*). Where this occurs on both, opposite flanks of a cell (e.g., Fig. 1 *j*) the phragmosome appears in section as a flat X. During interphase, cortical MTs are not excluded from this area of the wall along a three-way junction, yet during preprophase the condensing PPB MTs now avoid this region. PPBs do not form just anywhere within the broader zone bracketed by the X but either parallel to one of the limbs or between the tips of the X, forming a double PPB (see Fig. 2 *l*). The position of these slightly unusual PPBs is difficult to comprehend in terms of the placement of cortical MTs but is directly reflected by the junction of strands with the cortex. Factors affecting the positioning of strand alignment may therefore provide clues on band placement. Other recent work on stomatal complex formation also indicates that bunching up of cortical MTs provides no satisfactory explanation for the origin of PPBs that form 90° to the previous cortical array (Cleary and Hardham, 1989; Mineyuki et al., 1989; Cho and Wick, 1989). From the present studies, new sets of nucleus-associated MTs would be anticipated to be involved in forming such PPBs and this is currently under study.

Analogue Modeling of Strand Alignment

A major difference between orthogonal and nonorthogonal

cells is in the patterns formed by the nucleus-associated, radiating MTs. In elongated cells, an initially starlike arrangement realigns into a transverse baffle with minor polar strands at right angles. In cross section this constitutes the Maltese cross of Sinnott and Bloch (1940). In isodiametric cells approximating regular hexagons in section, the starlike array of strands persists. Both patterns can be mimicked by springs or bubble walls in a frame—the particular pattern depending upon the conformation of the frame. This is described more fully in the legend to Fig. 6. It demonstrates that elements under tension, capable of moving, seek a minimal path. In future work we will test the hypothesis that tension is an important part of the premitotic patterning of strands. However, Hahne and Hoffman (1984) have already elegantly demonstrated that living cytoplasmic strands do exert tension. They found that *Hibiscus* protoplasts were not smoothly spherical but had surface indentations. Cytoplasmic strands radiated from the central nucleus and, where they connected with the cortex, caused an in-pulling. Laser microsurgery of individual strands, or cytochalasin B treatment, removed these indentations, indicating that the strands had been under tension. Premitotic strands are also capable of sliding relative to the cortex. This was observed by Ōta (1961) in a study of dividing staminal hairs and we (Goodbody, K., and C. W. Lloyd, unpublished observations) have observed premitotic reorganization of strands in living *Tradescantia* epidermal cells.

Avoidance of Four-way Junctions

In sections of unwounded plant tissues only three walls meet at a point. This gives isotropically growing tissue a honeycomb appearance similar to that of bubbles in a foam (see Thompson, 1942). The basis of this fundamental aspect of tissue organization is some mechanism that inhibits a new cross wall from joining opposite the point on the mother wall where a cross wall between neighbors is already inserted. Sinnott and Bloch (1941) offered the tentative suggestion that if the phragmosome, at its point of attachment to the wall, carried on an exchange of nutrients, then the existing three-way junction could represent a “blind spot” somehow to be avoided. The analogue models suggest an alternative explanation. Elements under tension (strands, springs, bubble walls), in seeking a minimal path, automatically move away from the vertex of an angle. The angle necessary for the working of this avoidance mechanism appears at first sight to be absent from files of elongated cells where cross walls seem to contact neighboring long walls at 90°. However, these junctions are locally curved in an attempt to meet as three-way junctions of 120° separation.

A major exception where intercrossing, four-way junctions do form is in wound tissue. After wounding, new cross walls are inserted in a virtually synchronous manner often lining up across adjacent cells without staggering the joints (Sinnott and Bloch, 1941). In unwounded tissue, division is asynchronous and once a new cell plate has attached perpendicularly to an older side wall, the initial lack of expansion of the plate (Korn, 1980) causes the older wall to hinge into two angled facets during subsequent growth. This angling, necessary for the hypothesized avoidance mechanism, is absent when adjacent cells divide simultaneously after wounding.

In concluding, it would seem from our observations that there is some interaction between the alignment of premitotic strands and cell geometry which influences the division plane. It is clear, however, that other factors play a part since wound-induced division planes tend to parallel the line of the wound regardless of individual cell shape. The behavior of transvacuolar, microtubular strands during wounding is currently under study.

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