Organization of Microtubules in Centrosome-free Cytoplasm

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Abstract. Many different cell types possess microtubule patterns which appear to be polarized and oriented, in part, by cytoplasmic factors not directly associated with a centrosome. Recently, we demonstrated that cytoplasmic extensions ("arms") of teleost melanophores will reorganize their microtubule population outward from their centers after surgical isolation (McNiven, M. A., M. Wang, and K. R. Porter. 1984. *Cell.* 37:753–765). In the study reported here, we examine microtubule dynamics within the centrosome-free fragments and find that, after severing, microtubule reorganization is initiated at the proximal (cut) end of an arm and migrates distally with the aggregated pigment mass until it becomes permanently positioned at the middle of the arm. Computer-aided image analysis demonstrates that this middle position is located at the arm centroid, implicating the action of a cytoplasmic gel in this process. Morphological studies of arms devoid of pigment reveal that microtubules do not emanate from a single site or structure within the centroid area, but from a more generalized region. Taken together, these findings suggest that factors distributed throughout cytoplasm participate in microtubule assembly and organization.

THE mechanisms by which eukaryotic cells regulate the number, polarity, orientation, and spatial distribution of their microtubules are poorly understood. It is believed that specific central structures, such as centrosomes, spindle poles, or basal bodies provide an essential environment for the initiation of tubulin assembly and organized microtubule growth (for reviews see Raff, 1979; Tucker, 1979; Brown et al., 1982; McIntosh, 1983). Although these nucleating structures undoubtedly play an important role, it has become apparent that more peripherally placed cytoplasmic constituents are also involved in both the assembly and disposition of microtubules. An example of this peripheral influence can be found in the scleroblasts located on the fibrillary plate of teleost scales. When stained with antibodies to tubulin and viewed in situ, the microtubules are seen to extend out radially from the cells' organizing centers, and then curve abruptly to become aligned with the collagenous fibers of the underlying scale. It is impressive that microtubules in adjacent cells are similar in number, all oriented in the same manner, and all share a nearly identical pattern (Byers et al., 1980). It seems highly unlikely that the centrosome alone can induce changes in the direction of microtubule growth from a distance of many micrometers without the aid of other organizing components situated in peripheral cytoplasm. In addition, there are cell types in which cytoplasmic microtubules are not associated with an obvious nucleating structure, as in the epidermal cells of the developing leg tarsomere of the blowfly. In these cells there are two different microtubule populations. The first exhibits conventional radial growth from a centrosomal complex of dense bodies, while the second consists of a cortical layer of microtubules that is independent of the centrosomal microtubule population (Tucker, 1979). Perhaps the most graphic demonstration of a cell's ability to organize microtubules independent of a centrosome can be found in elongating axons. Despite the fact that axoplasm may extend outward over great distances from the centrosomal complex of the perikaryon, microtubules are ordered in their arrangement and uniformly polarized. A substantial number of studies have examined the length, number, and continuity of axonal microtubules to determine whether individual microtubules can span the entire nerve fiber. The findings indicate that microtubules are not continuous structures but show numerous starts and stops over the length of the axon (Nadelhaft, 1974; Bray and Bunge, 1981; Sasaki et al., 1983). In fact, the peripheral axon may possess more microtubules than are found within the cell body itself (Zenker and Hohberg, 1973). These observations suggest that an axon is capable of initiating microtubule assembly at points along its length, quite independently of a single centrosome. An alternative view, which should not be discounted, is that microtubules first nucleate and grow from a centrosome within the cell body, later become fragmented, and then are translocated outward into the distal regions of the axon. This model is supported by the fact that tubulin is a major component of slow axonal transport (Hoffman and Lasek, 1975; Black and Lasek, 1980).

Recently, we set out to test whether peripheral cytoplasm within cells can assemble uniformly polarized and well-ordered microtubule bundles in the absence of a centrosome. For several reasons, the teleost melanophore is a favorable cell model for such a study. These neuronally derived cells exhibit rapid intracellular pigment movements in response to caffeine and epinephrine and, most importantly, they have a stellate morphology with many dendritelike branches or
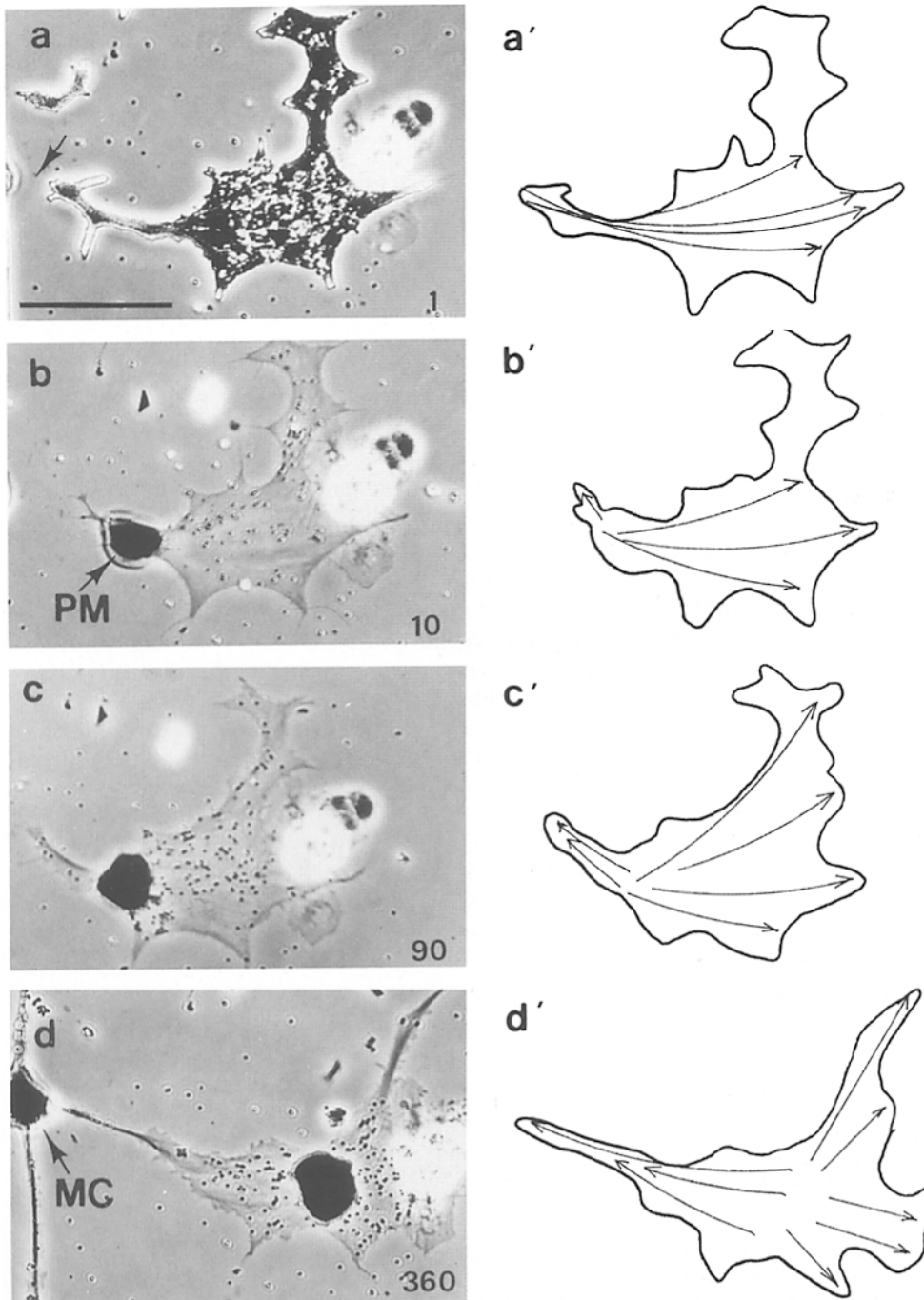
Figure 1. The point to which a severed melanophore arm aggregates pigment will migrate from the cut to the arm center over time. A phase microscopy series of a severed melanophore arm which was stimulated to aggregate and disperse its pigment, repeatedly, over a 6-h period after surgical isolation. (*a*) 9 min after severing, the pigment granules are dispersed throughout the arm. Upon addition of epinephrin, most of the pigment aggregates unidirectionally toward the cut end (*arrow*), forming a large, round pigment mass (*PM*). When stimulated at later time points (90 min), the pigment aggregates to a more distal location (*c*). The point to which pigment aggregates will continue to migrate away from the cut until it eventually reaches the arm center 6 h later (*d*). *a'–d'* are illustrations depicting the changes in orientation and polarity of the microtubule population within the severed arm pictured in *a* and *b*. Each thin line represents a single microtubule while the arrowheads denote the "plus ends." *MC*, main cell. Numbers in micrographs indicate time in minutes after severing. Bar, 30 μm.

arms extending outward for long distances (50–200 μm) from the cell body. Numerous microtubules, emanating from a large centrosomal complex located within the cell body, extend (with plus ends out) into the arms to form a cortical microtubule "cage" (Schliwa, 1978). With a glass microneedle, one can surgically isolate the cytoplasmic arms and their microtubules from the melanophore cell body. We found, unexpectedly, that >4 h after isolation, severed arms changed the original polarity and orientation of their microtubules. By the end of the incubation period, microtubules reorganized with their minus ends gathered into the arms' centers and plus ends oriented toward the plasma membrane, as if to

form new microcells (McNiven et al., 1984). After this major alteration in microtubule organization, there comes a reversal in the direction of pigment transport in response to caffeine or epinephrin. Rather than transporting pigment to and from the cut end, which is the case immediately after severing, an arm aggregates pigment to and disperses it from an area near its center (see Fig. 1). In a separate study using taxol, we have shown that pigment will not aggregate to the center of a severed arm until the microtubule population has reoriented, and that the direction a pigment granule moves is dependent upon the polarity of the associated microtubules (McNiven and Porter, 1986). Thus, over time, these cyto-

plasmic fragments establish what we have termed "a new functional center," which performs the same role as a centrosome in the intact melanophore.

In this study, we examine the mechanisms of microtubule growth and reorganization in the cytoplasmic fragments of melanophores. We find that these fragments, or arms, can recover completely from treatments with cold and microtubule poisons to regrow a properly oriented microtubule population from a new center. This recovery will occur in arms without pigment granules, and is therefore independent of pigment and associated matrix proteins. Most importantly, microtubule regrowth within severed arms is not from a traditional centrosomal complex or organizing center, as appears to be the case in whole melanophores, but is from a more generalized concentration of matrix elements located at the severed arm center. Using a computer program, we show that this center, in addition to being the region to which pigment aggregates, is a cytoplasmic centroid. We believe that the process of reorganization that occurs after the severing of a melanophore arm involves the action of a dynamic structural continuum or gel, and demonstrates that cytoplasm as a whole possesses an innate ability to both nucleate and guide microtubule assembly in the absence of any conventional nucleating structures.

Materials and Methods

The techniques for melanophore culturing, electron microscopy, indirect immunofluorescence, and the microtubule hook assay were all described in detail in an earlier paper (McNiven et al., 1984).

Determination of the Cytoplasmic Centroid

To obtain digitized coordinates, electron or light micrographs of six different severed arms were enlarged and photographically printed. The periphery of each photographed arm was translated into ~50 digitized points using a model No. 1224 digitizer (Numonics Corp., Landsdale, PA). These coordinates were then used by our campus VAX computer with a program designed by Statistical Services (a subsidiary of the Mathematics and Computer Science Department at University of Maryland, Baltimore County) to calculate the centroid of each asymmetric shape representing an arm. The program was tested on standard geometric shapes such as rectangles, circles, and squares to prove its accuracy. Because our computer program uses digitized coordinates from two-dimensional photographic images, we have defined "centroid" as the precise center of a given cytoplasmic area. It is more biologically relevant to express the centroid as a point fixed within a three-dimensional space, and thereby view it as a center of a cytoplasmic mass rather than area. To do this one would need to establish that the depth, or Z axis, of a severed arm is relatively uniform. Therefore, we first examined, with electron microscopy, the cross-sectional profiles of 10 different severed or intact arms containing dispersed pigment. We found repeatedly that although the cross-sectional heights varied from arm to arm (1–3 μm), the distance between the top and bottom plasma membranes within a single arm was remarkably consistent (deviation <0.25 μm). We regarded this variation minor enough to consider the melanophore arm a laminar structure. This assumption is not absolute, however, because we did not examine profiles from the same arms that were subjected to centroid analysis.

Disassembly and Recovery of Microtubules in Severed Arms

We have found that microtubules in teleost chromatophores are, unfortunately, exceedingly stable. Therefore, it was necessary to find the combination of cold and drug required to disassemble completely the microtubules without killing the severed arms. The procedure that filled both of these criteria is as follows. Melanophore arms, emptied of pigment, were first surgically severed from their parent cells, and then allowed to reorganize their microtubules and form new centers over a 5-h incubation period. Next, the

arms were placed in a 4°C refrigerator for 10 min to allow a gradual decline in temperature. The arms were then exposed to cold (4°C) media containing 0.5 mg/ml of nocodazole (Sigma Chemical Co., St. Louis, MO) for 20 min, and placed back into the 24°C incubator, while still in drug media, for 2 h. At the end of this time, neither severed arms nor intact cells contained any microtubules when inspected by indirect immunofluorescence with tubulin antibodies or by electron microscopy. Experimental arms were then rinsed six times in drug-free media and allowed to recover for different time periods before lysis for the hook assay or fixation for immunofluorescence.

Results

Site of Initiation for Microtubule Reorganization in Severed Arms

The fact that melanophore arms isolated from a parent cell can reorganize microtubules outward from their centers was demonstrated earlier (McNiven et al., 1984) through the use of a microtubule polarity or "hook assay" developed by Heidemann and McIntosh (1980). Numerous applications of this assay have been described in detail elsewhere (Euteneuer and McIntosh, 1981; Heidemann et al., 1981). Using the hook assay, we first studied the polarities of microtubules at time points between arm severing and the formation of a new center. We asked what happens to the microtubule population in vivo when its free minus and plus ends are exposed to the cytoplasmic milieu, as when the arm is first severed. Is the new center suddenly assembled in the middle of the arm several hours after severing, or is it formed gradually? If the latter is true, what are the intermediate steps? Fig. 1, a–d is a series of phase-contrast photomicrographs depicting a single, isolated melanophore arm that was stimulated to aggregate pigment at different times after severing. At 10 min after isolation, when exposed to epinephrine, the arm aggregates pigment retrograde into an area near its cut end (Fig. 1 b). Pigment also recedes from a small region of cytoplasm proximal to the cut. When the arm is returned to the culture incubator, allowed to redisperse its pigment, and then stimulated to reaggregate pigment at later time points, the pigment aggregates to a more distal location progressively further from the cut end (Fig. 1, c and d). By 6 h, pigment aggregates to a point near the arm center and does not migrate any further despite longer incubation times.

We examined the polarity of microtubules in other comparable arms, like the one shown in Fig. 1 b, that would also aggregate pigment to intermediate points between the cut end and the center after severing. We found that ~90% of the microtubules within the two arms assayed have their minus ends located within the region occupied by the pigment mass (Table I). Likewise, microtubule plus ends were found to extend outward from the pigmented region to the peripheral arm cortices. Thus, the aggregated pigment mass and the area of presumptive microtubule regrowth occupy the same cytoplasmic location and together migrate distally over time from the arm's cut end to its middle.

A Severed Arm Positions Its Newly Formed Functional Center at Its Cytoplasmic Centroid

As mentioned earlier, a newly formed functional center within a severed arm will migrate away from the proximal cut end into a more central region of the arm, but does not continue out into the distal arm tip. This region is not necessarily a point equidistant between the proximal and distal

Table I. Microtubule Reorganization Migrates from an Arm's Proximal to Distal End after Severing

Cell	CW microtubules	CCW microtubules	CW	CCW
	n	n	%	%
A Proximal	6	61	9	91
Distal	59	8	88	12
B Proximal	9	64	12	88
Distal	31	3	91	9

Polarities of microtubules from cells examined 90 min after surgical isolation as in Fig. 1 c. Proximal represents the region of the arm residing near or proximal to the cut. Distal represents the region of the arm furthest from or distal to the cut. CW, clockwise; CCW, counterclockwise.

Table II. Microtubules' Minus Ends Are Gathered at the Center of the Larger of Two Connected Arms after Severing

| Cell	CW microtubules	CCW microtubules	CW	CCW
			%	%
A Distal L	31	1	97	3
Distal S	33	3	92	8
Proximal L	3	78	4	96
Proximal S	30	2	93	7
B Distal L	95	31	75	25
Distal S	30	2	94	6
Proximal L	4	149	3	97
Proximal S	21	1	95	5

Microtubule polarities from two different severed arms each possessing a large (L) and smaller (S) branch. 5 h after severing, upon addition of epinephrin, all pigment aggregated into the large branch as illustrated in Fig. 2 g. Proximal represents the region of the arm residing near or proximal to the cut. Distal represents the region of the arm furthest or distal to the cut.

arm ends but appears to be the central point of a fragment's cytoplasmic area. One could predict that this location contains the greatest amount of bulk cytoplasm and is somehow involved in determining the position of the newly formed functional center. A further demonstration of this is found in unusually shaped, asymmetric arms which possess two branches, one large and one small (Fig. 2 a). Using these branched arms we were interested in finding where a new functional center would be established; i.e., in each branch, at the vertex between the branches, or within the larger branch only. We find that when a branched arm is stimulated to aggregate pigment 5 h after surgical severing (Fig. 2 b), pigment translocates out of the distal, proximal, and smaller branched regions of the arm into the largest branch (Fig. 2, c and d). The result is a large single mass of pigment positioned at the center of the large branch (Fig. 2, e and g). Examination of the microtubule population within this arm, using indirect immunofluorescence (Fig. 2 f), suggests that the microtubules in each branch are organized differently. Although the microtubules in the small branch are distributed in the normal linear pattern exhibited in uncut arms, the microtubules in the larger arm are less linearly organized, and appear to extend outward in all directions from the pigment mass. These contrasting patterns suggest that microtubules in the larger branch of the arm undergo a reorganization while those in the smaller branch do not. We analyzed other, similarly shaped, branched arms with the polarity hook assay to determine the orientation of the microtubules in both branches and confirm whether the large branch does indeed reorganize its microtubules outward to form a new functional center. Table II lists the number of clockwise and counterclockwise microtubules in the different regions of two different branched arms. Fig. 2 g shows one of these arms (arm A in Table II) with both its large and small

branches containing hooked microtubules. The arrows from each electron micrograph (Fig. 2 g) point to the regions of the arm where the sections were taken. In both arms examined, it is clear that a repolarization of microtubules occurs only within the proximal regions of the large branches (an average of 96% read counterclockwise) and not in the small branches (an average of 94% read clockwise). Fig. 2 f shows the organization and polarity of these microtubules. Thus the polarity assay supports our interpretation of the immunofluorescent images and indicates that microtubules reorganize outward from the middle of the larger arm branch. Again, the largest cytoplasmic region of a fragment appears, by its size, to play a role in positioning the new center and determining which arm areas will reorganize their microtubules.

We have thus far referred to the concept of a cytoplasmic center in a qualitative way. It seemed important to measure this phenomenon and determine with a greater degree of certainty whether the new functional center occupies the centroid of a fragment. To this end, we subjected the photomicrographs of six different severed arms, which had established new centers, to computer-aided image analysis (see Materials and Methods). The periphery of each arm was translated into digitized circumferential coordinates which were in turn used by a computer programmed to determine where the center of area, or centroid, of each fragment was located. The position of each arm centroid, marked with cross hairs, could then be compared to the position of its functional center, visualized as an aggregated pigment mass. Fig. 3 shows phase micrographs of four different severed arms (Fig.

Figure 2. Pigment aggregates from the smaller into the larger cytoplasmic areas of branched severed melanophore arms. A phase-contrast, light micrograph series of pigment aggregation in a severed arm possessing two branches, one large (L) and one small (S). After severing with a glass microneedle, the isolated, pigment-filled arm was returned to the culture incubator for 5 h (a), then stimulated to aggregate pigment. (b-d) Pigment migrates out of the smaller arm branch, around a bend, and into the center of the larger branch, forming a single spherical pigment mass (PM). (c) The wavy, white arrow depicts the direction of translocating pigment. (e) The same branched arm stained with tubulin antibodies. Note that microtubules in the large branch appear to extend outward from the pigment mass while microtubules in the smaller branch appear linear and unchanged. (f) Line drawing depicting the polarity of the microtubule population in the same arm. Despite the arm's irregular shape, the microtubules are oriented properly to insure homogeneous retrograde and orthograde pigment transport during aggregation and dispersion, respectively. (g) Microtubule polarities from a severed arm, that possessed a large (L) and smaller (S) branch. 5 h after severing, upon addition of epinephrine, all pigment aggregated into the large branch. Electron micrographs show "hooked" microtubules present in the different arm regions (arrows). Bar, 30 μm.
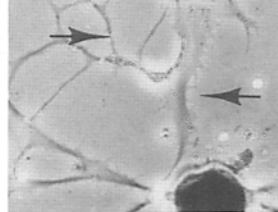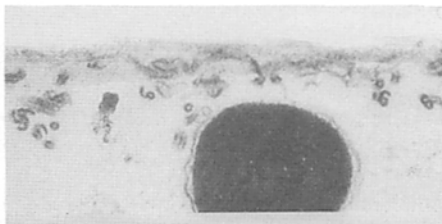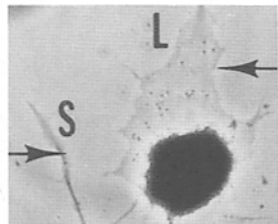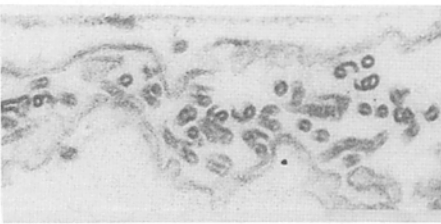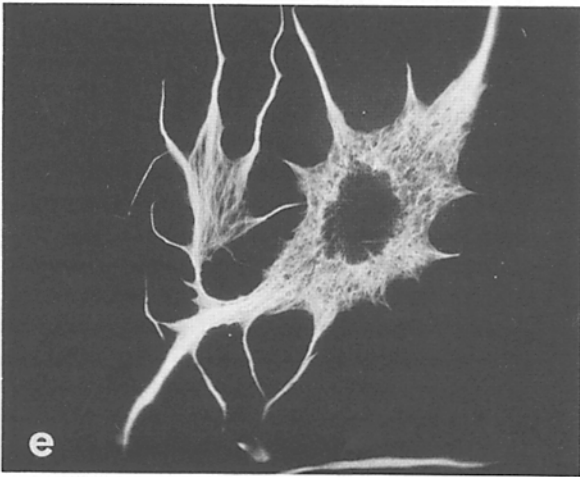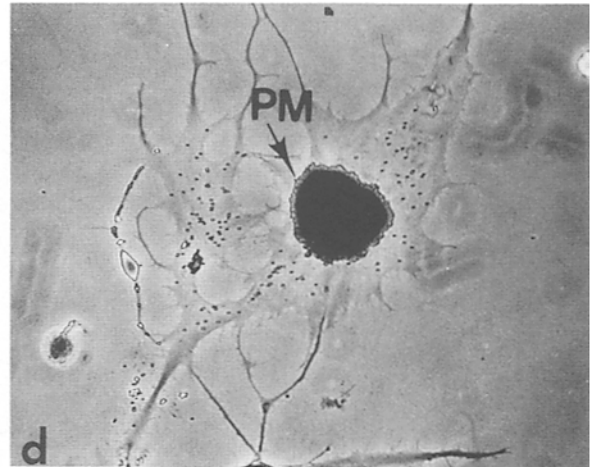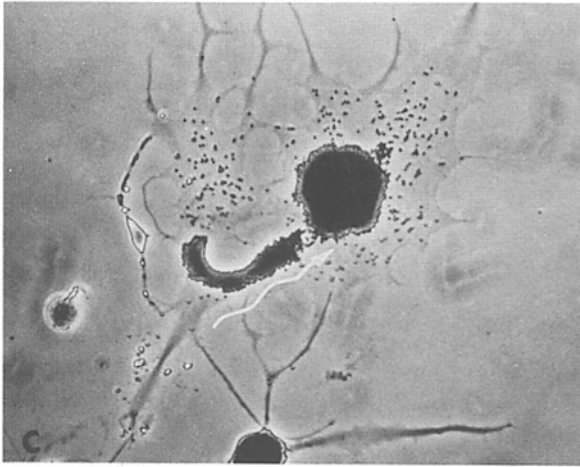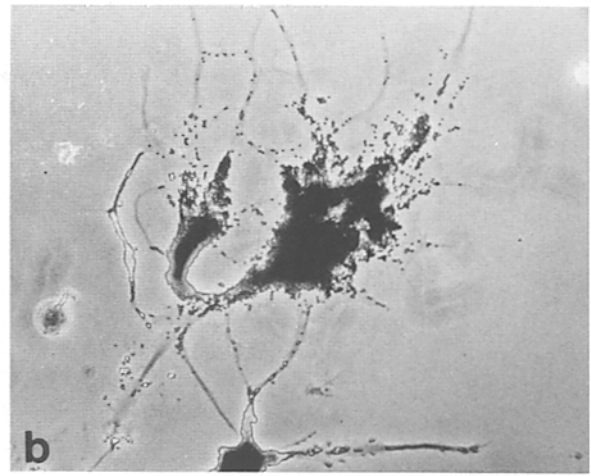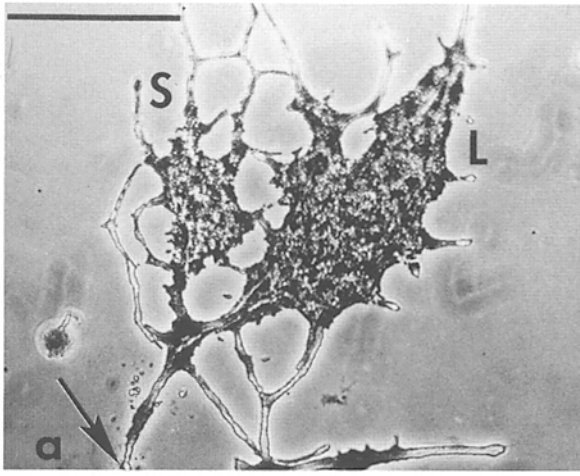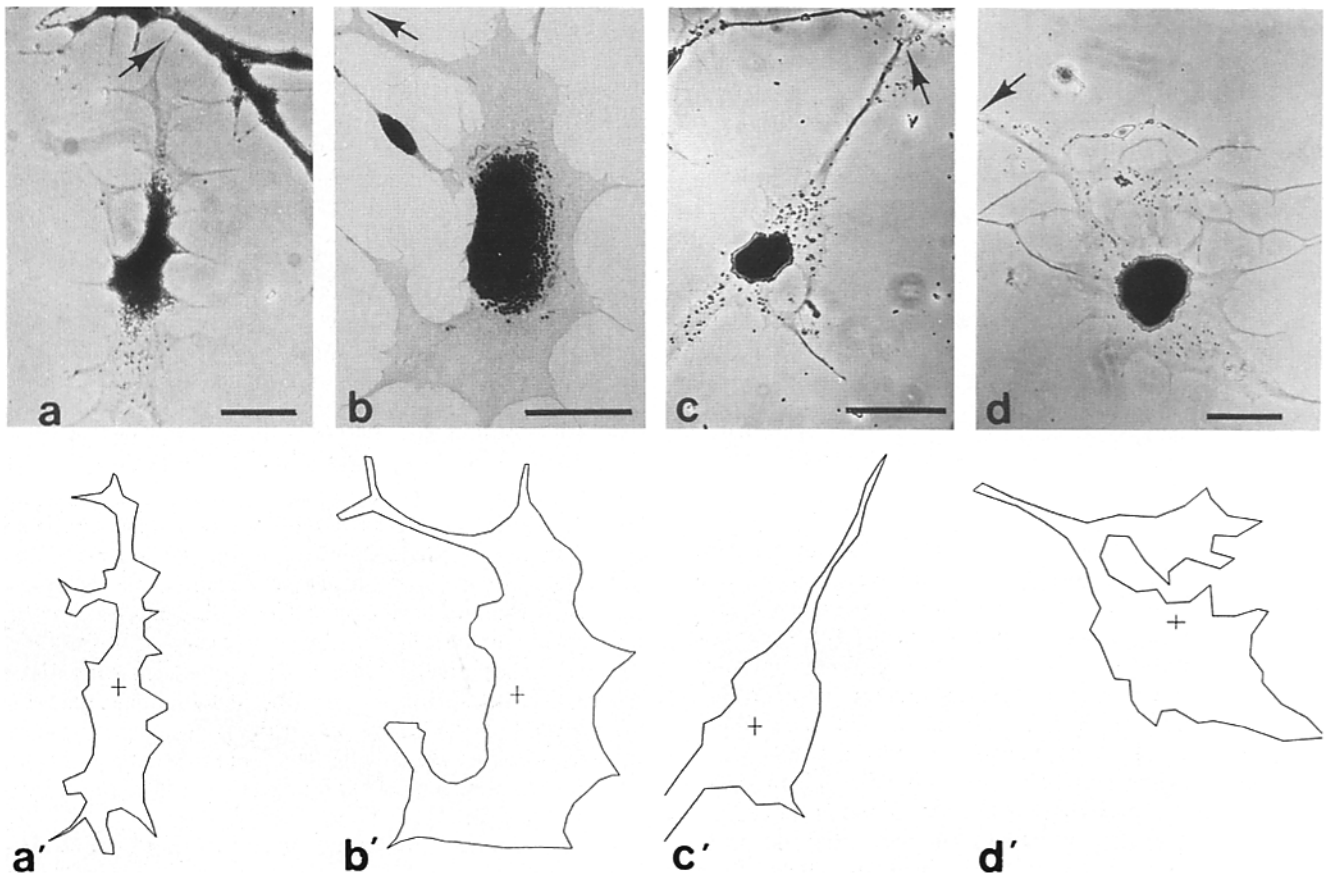
Figure 3. Severed melanophores arms aggregate pigment to, and extend microtubules from, their cytoplasmic centroids. (*a–d*) Light micrographs of four different melanophore arms which have reorganized their microtubules and aggregated pigment into their centers. The arrow depicts the cut end of each arm. (*a'–d'*) The circumference of each arm was outlined, digitized, and used to calculate the cytoplasmic center of area, or centroid, which is indicated by a +. Note that the calculated center in each arm corresponds remarkably well with the point to which pigment aggregates. Bars, 20 μm.

3, *a–d*) which reorganized their microtubule populations and aggregated pigment into their centers. Beneath the micrographs are the corresponding line drawings of each arm (Fig. 3, *a'–d'*) with centrally situated cross hairs representing the computer-generated centroids. Remarkably, the cross hairs in each case are situated over the center of each pigment mass with near precision. Even in situations where the pigment mass has favored one side of the arm (Fig. 3 *b*), or a large branch over a small branch (Fig. 3 *d*), the cross hairs and the center of the aggregated pigment mass occupy the same location. Thus, after surgical isolation, fragments reorient their microtubules around and aggregate pigment to their cytoplasmic centroids.

Microtubules in Severed Arms Emanate from a Generalized Region around the Arm Centroid and Not around a Single Nucleating Structure

It seemed possible that a new functional center located at an arm's centroid would represent the formation of a nascent centrosome-like structure. Such a structure might be devoid of centrioles and consist merely of the amorphous material or dense bodies (microtubule-organizing centers) which

comprise part of the centrosomal complex of whole intact cells. Obviously, the presence of pigment within the severed arms would make any morphometric search for such a nascent center uninformative. To circumvent this problem, we examined the polarity of microtubules in arms which, before severing, had been stimulated to aggregate their pigment into the parent cell. With these pigment-free or empty arms, we first investigated whether the reorganization and repolarization of microtubules is dependent upon the presence of pigment and/or their associated cytomatrix proteins. If not, we could then discover whether an arm center unobstructed by pigment would facilitate our search for any kind of putative microtubule nucleating site or structure. We found first that pigment-free arms will reorganize their microtubules over time. As demonstrated in Table III, the three severed arms, examined by the microtubule polarity hook assay, reorganized >87% of their microtubules outward from their centers, a percentage similar to that found in severed arms containing pigment (McNiven et al., 1984). We conclude, therefore, that isolated melanophore cytoplasm is competent to reorganize its microtubules in the absence of pigment granules and associated proteins. With this information in hand, we examined the unobstructed centers of arms in search of sites

Cell	CW microtubules	CCW microtubules	CW	CCW
			%	%
A Proximal	13	108	11	89
Distal	13	1	93	7
B Proximal	37	150	20	80
Distal	82	3	96	4
C Proximal	8	100	7	93
Distal	NC	NC	–	–

Polarities of microtubules from arms which were emptied of pigment, then severed and incubated for 5 h before assay. Proximal represents the region of the arm residing near or proximal to the cut. Distal represents the region of the arm furthest or distal to the cut. *NC*, none counted.

from which microtubules may grow. Fig. 4 shows electron micrographs of a pigment-free arm that was returned to the culture incubator for 5 h after isolation, then fixed, embedded, and serially sectioned parallel to the substrate. Low magnification electron micrographs (Fig. 4 *a*) show mitochondria, microtubules, and a large quantity of smooth endoplasmic reticulum (SER).[1] When viewed under higher magnification (Fig. 4 *b*), the microtubules are more easily resolved and appear as well-ordered bundles which show a preferred association with the cortices of the arm branches. These microtubules may either terminate at the base of the extremity, continue along the arm cortex, or extend into the arm center where they become mixed and unorganized before finally terminating. The most important point to note, from this and the many other sectioned arms we have examined, is that microtubules do not terminate at a specific site but throughout the central region of the arm normally occupied by the aggregated pigment mass. This corresponds with our sectioning–polarity data showing that the majority of the microtubule minus ends are gathered within the aggregated pigment mass. It is worth noting that the SER is largely restricted to the arm center, with only a few cisternae extending out into the peripheral branches. Since the SER within intact arms is usually evenly distributed, it appears that the SER has receded from the periphery of the arm to accumulate at a central region. The majority of the mitochondria are also centrally located and seemingly excluded from the peripheral regions of the arm.

When pigmentless arms are fixed, dried by the critical point method, and viewed as whole-mount preparations with a high voltage electron microscope, it becomes obvious that the SER, mitochondria, and cytomatrix components accumulate in the arm center after severance. The apparent redistribution of these components is quite dramatic when compared to unsevered melanophore arms prepared the same way. Fig. 5 shows high voltage electron micrographs of two different melanophore arms, one arm still attached to the parent cell (Fig. 5, *a* and *b*) and one severed from the parent cell (Fig. 5, *c* and *d*), then incubated for 5 h. In the intact central arm, the distribution of both cytoplasmic matrix and membrane components is even throughout. In the severed arm, these components are gathered within the central re-

gion as if they have receded from the arm extremities. Numerous filamentous strands interconnect membranous tubules of the SER to form dense foci while the peripheral cytoplasm appears relatively thin and empty (Fig. 5 *c*, *asterisks*). The microtubules, which can be seen as linear bundles extending through the central region of the intact arm, appear randomly oriented in the center of the severed arm (Fig. 5 *d*).

To obtain a more generalized overview of the microtubule population within an arm, and to search the arm center for any aster-like nucleating structures normally found in intact, whole cells, we subjected pigment-free severed arms to indirect immunofluorescence using tubulin antibodies. Fig. 6 contains a phase-contrast micrograph showing two different empty, severed arms (Fig. 6 *a*) and the corresponding fluorescent micrograph (Fig. 6 *b*). The fluorescent images support our electron micrograph observations and reveal that the microtubules are organized differently in the center of an arm than at its extremities. In the broader regions of the arms, the microtubules intersect to form a tangle. Microtubules that extend into the long axonal-like processes of an arm appear to become laterally associated to form well-ordered linear bundles. We know from sectioning data, gathered from this and previous studies, that the vast majority of microtubules within these arm processes are oriented homogenously with plus ends reaching outward, whereas the disoriented microtubules within the arm centroid are of mixed polarities.

In a final search for nucleating sites, we subjected severed arms to low temperatures and microtubule poisons to disassemble their microtubules and follow their subsequent regrowth during recovery. We asked whether the arms could regrow a properly oriented microtubule population after the relatively harsh treatment of drugs and cold and, if so, whether regrowth would occur within the arms' centers to form characteristic asters. Such a study is depicted in Fig. 7, which shows three different arms that were emptied of pigment, severed, incubated for 5 h to allow microtubule reorganization, treated with nocodazole and cold, and then fixed at different time points during recovery in drug-free media (see Materials and Methods). The immunofluorescent micrographs represent cells fixed after 30, 60, and 120 min of recovery, respectively. Assembled microtubules are first visible within the narrow extensions of the arms (Fig. 7 *a*, *arrow*), and are not present within the arm bodies until later time points (Fig. 7 *b*, *arrows*). After 2 h of recovery, a full complement of well-organized and normally polarized microtubules can be seen filling the arms (Fig. 7 *c*). The results of a polarity assay conducted on arms recovered from drug and cold treatment (Table IV) show that the reassembled microtubules are properly oriented with >84% of their plus ends extending outward into the peripheral processes. The polarity of this reassembly, while less homogeneous than that observed in the examined untreated severed arms (Tables I–III), is more uniform than that found in an intact arm still attached to its parent cell treated the same way (Table IV).

Discussion

Dynamics of Free-ended Microtubules In Vivo

One of our initial objectives in using isolated melanophore

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1. *Abbreviation used in this paper*: SER, smooth endoplasmic reticulum.
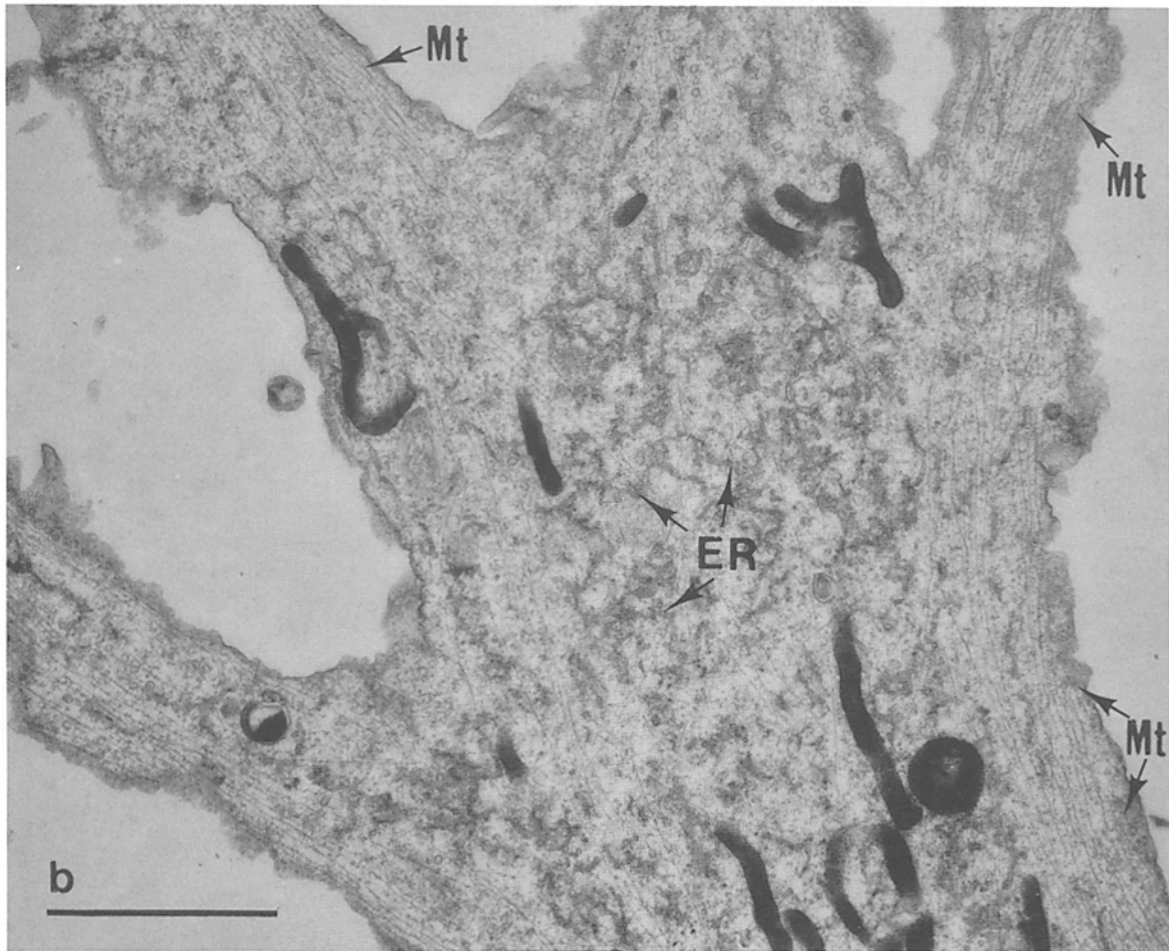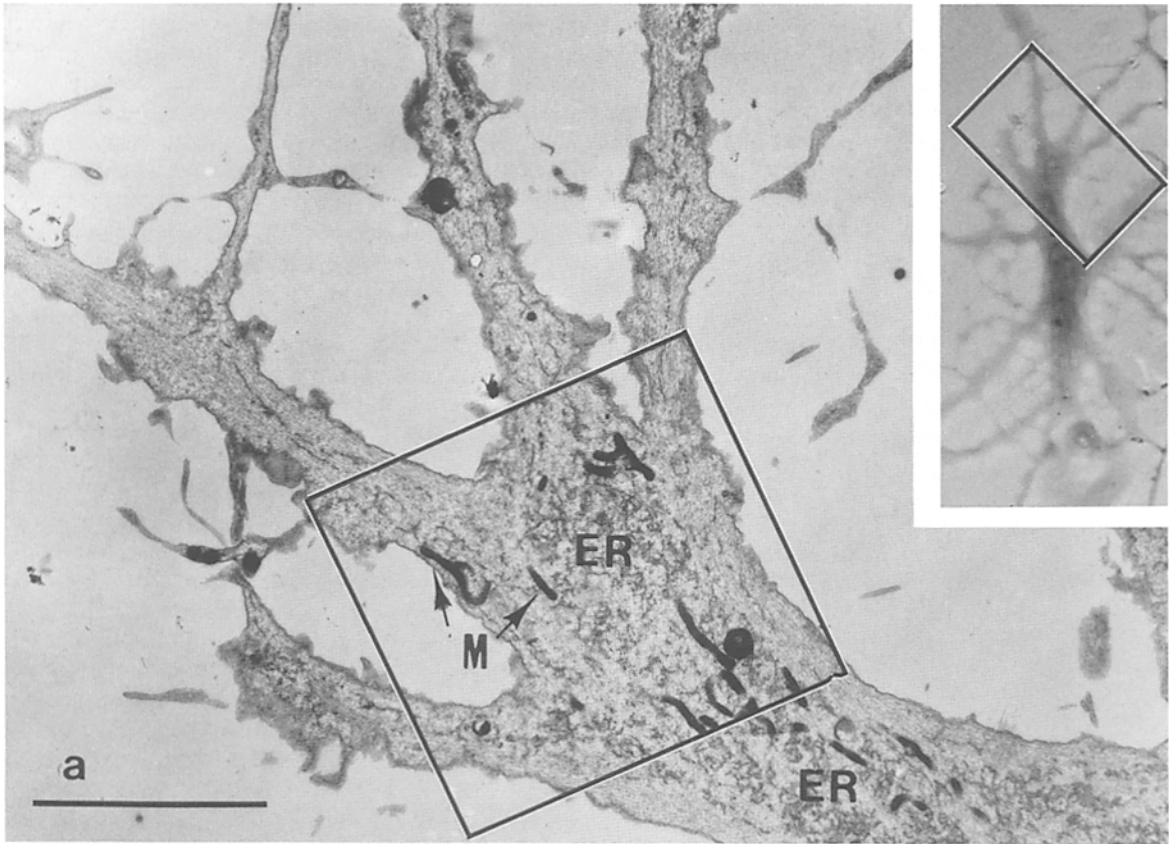
fragments was to examine the in vivo dynamics of a microtubule population severed from its centrosomal association. From time course experiments (Fig. 1), we can conclude that the formation of a "new functional center" within a severed arm is not a spontaneous event but is initiated at the arm cut and migrates, over time, to become situated at the central region of the arm. The adjacent line drawings in Fig. 1, $a'-d'$, illustrate in simplified form the orientation and polarity of the microtubule population in the photographed cut arm, and show that the microtubules' minus ends share the same location over time as the migrating pigment mass. We know for several reasons that the microtubules behave in this manner. First, it has been established from a previous detailed study, using the microtubule-stabilizing drug taxol (McNiven and Porter, 1986), that aggregation of pigment into the center of an arm cannot proceed without the microtubules first reorienting. Second, hook assays conducted on more than 15 different severed arms all show that the point to which pigment aggregates and from which microtubules emanate is the same. Third, we have hook assayed the polarity of microtubules in arms like the one shown in Fig. 1 c, which is in the process of cytoskeletal reorganization 90 min after surgical isolation. We find that the structural polarities of an arm's microtubules are different on opposite sides of the pigment mass, thereby confirming the belief that the locations of the microtubules' minus ends change along with the migrating pigment mass. We have termed this area the "new functional center," not necessarily because of its eventual position in an arm but rather due to its performing the role of the conventional centrosome found in intact cells.

From the criteria described above, it appears that microtubule regrowth is initiated within the region of the cut end after severing. As the length of newly formed microtubules increases, the position of the new center moves distally; although we can just as correctly say that as the new center moves distally the length of newly formed microtubules increases. From this study, we cannot determine which ends of the microtubules undergo subunit addition and loss. The fact that formation of the new center is initiated at the arm cut suggests that disassembly occurs at the microtubules' minus ends, newly exposed by the severing process. However, another plausible alternative is that the microtubules within a melanophore arm are dynamically unstable and undergo a random and rapid disassembly from their plus ends (Mitchison and Kirschner, 1984). When severed, each microtubule would proceed to disassemble by subunit loss from its plus end, while concomitant growth of new microtubules would be initiated near the arm cut.

## Implication of a Cytoplasmic Gel in Microtubule Organization

Whatever the precise mechanisms involved in the disassem-

bly and reassembly of microtubules after arm severing, it is quite clear that the cell fragments control the position of their newly formed functional centers. Despite the presence of extra cytoplasmic appendages or branches, an arm can selectively reorganize its microtubule population within a specific area and thereby aggregate pigment to that region when so stimulated. This is demonstrated within branched arms that reversed the polarity of their microtubules exclusively in the proximal regions of their largest branch (Fig. 2, Table II). In doing so, the arm maintained a properly oriented population of microtubules with minus ends gathered at a single region (the new center) and plus ends extending outward to the periphery, thereby eliminating any potential confusion as to where pigment should aggregate to and disperse from (Fig. 2 f).

One is confronted with such questions as (a) what factors within an isolated arm are involved in determining where a reorganization of microtubules should or should not occur, and (b) how is the final position of the new center determined? The computer-aided image analysis we have conducted has enabled us to locate the centroid within six different severed arms; the position of the centroid coincides with that of the new functional center (Fig. 3). The overlap of the computer-generated cross hairs and the central pigment mass is too consistent to be coincidental. When the pigment mass is set off to one side of an arm, as in the arms in Fig. 3, b and d, the calculated centroid is also. From this striking correlation, it would appear that factors or forces within a severed arm strongly favor this positioning and that this centroid location is the region in which tension forces exerted on the new center from all regions of the arm are equal. Indeed, one may interpret the migratory behavior of the new center as its attempt to find a stable position like that occupied by a centrosome within an intact cell. It has been well documented by Bray (1979) that cells are subject to pulling forces and will subsequently alter their morphology when such forces are thrown into an imbalance. Bray calculated the amount of tension that a neurite exerts upon its parent cell center and consistently found that a neurite pulling on one side of a cell body was always counterbalanced by a neurite of appropriate size on the other side. If several neurites were amputated from a single cell, the cell would respond in turn by repositioning its remaining neurites or forming new ones, resulting in an eventual return to free equilibrium. In contrast to neurons, melanophore arms may respond to the sudden force imbalance generated through severing by forming a new center at the arm centroid rather than undergoing extensive cytoplasmic regrowth. The end result is the same, however: an eventual equilibration of initially unbalanced pulling forces.

Within a melanophore, we find two probable candidates which could act to position a new center within an arm, the

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Figure 4. Ultrastructure of a severed melanophore arm center devoid of pigment. (a) Low magnification electron micrograph of an arm which aggregated pigment into the mother cell and was then severed, incubated for 5 h, fixed, embedded, and longitudinally sectioned. A low magnification light microscope image of the entire arm is provided in the inset. The endoplasmic reticulum (ER), which is evenly spread throughout an intact arm, has receded from the peripheral neuritelike extensions and gathered in the arm center. (b) A higher magnification of the boxed region in a shows that the microtubules (Mt) do not emanate from any single focal point but instead appear to be aligned along the plasma membrane and travel out into the peripheral extensions where they become extremely well organized. The large quantity of endoplasmic reticulum at the arm center is plainly seen. M, mitochondria. Bars: (a) 5 μm; (b) 2 μm.
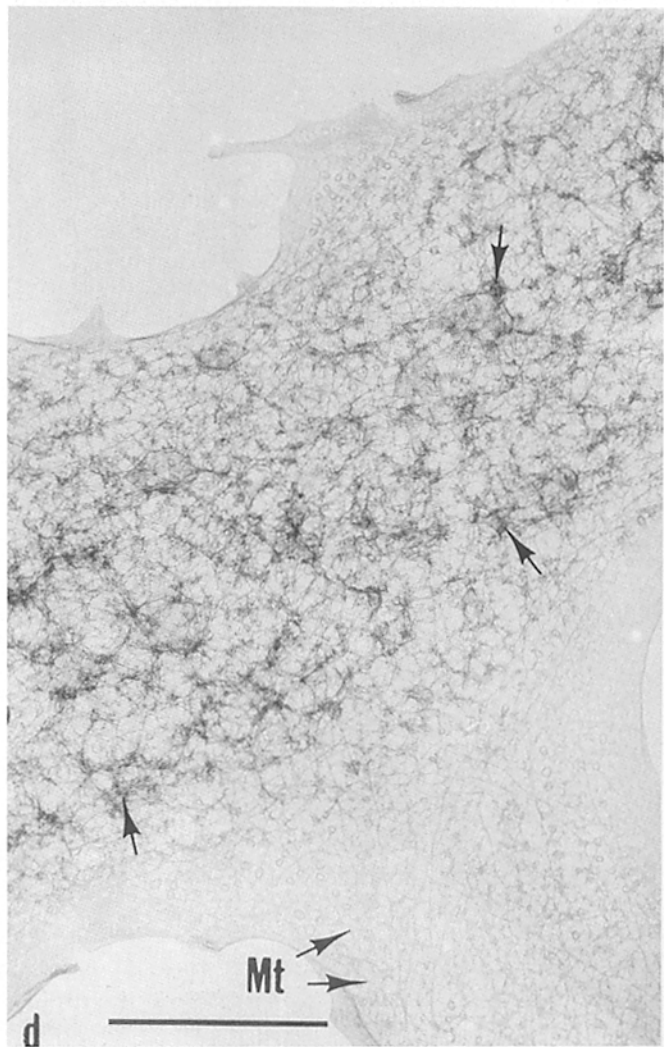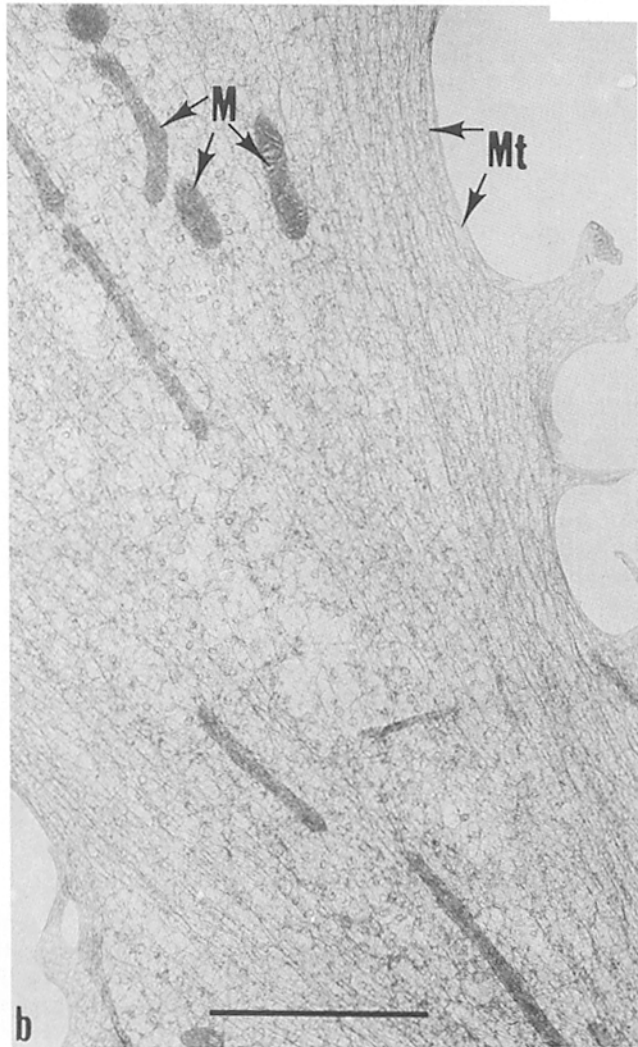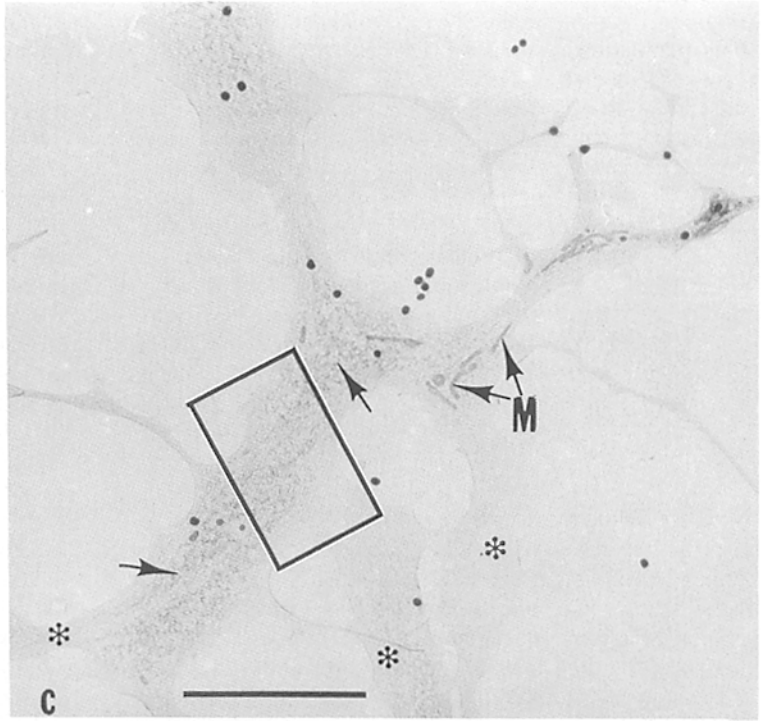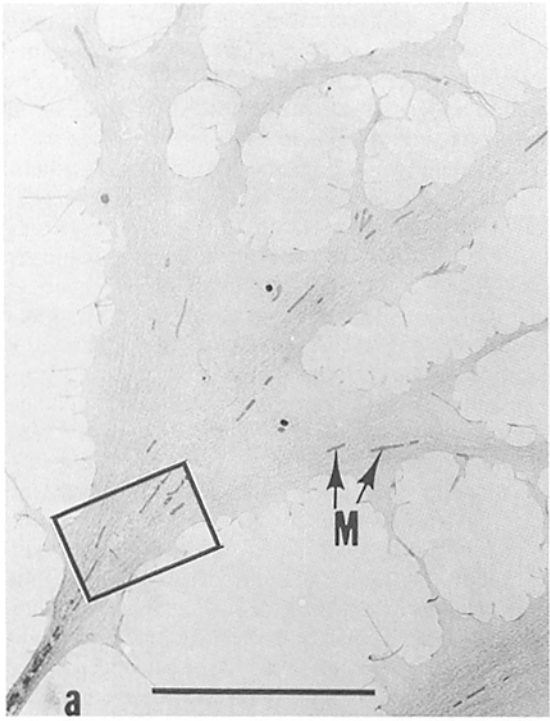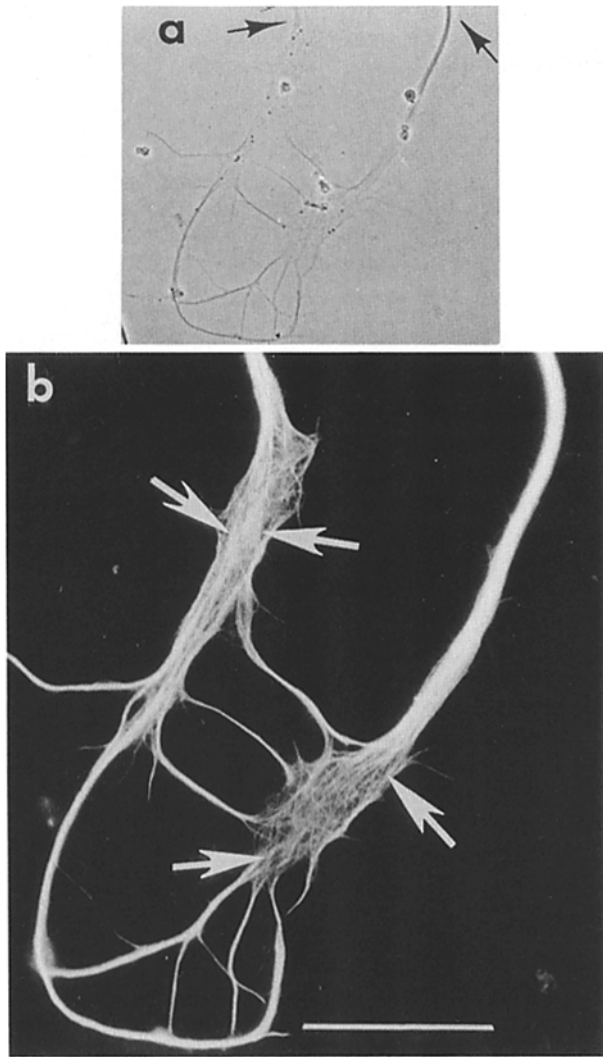
Figure 6. Organized microtubules in severed arms do not extend from a common nucleating site. Phase-contrast and fluorescent micrographs showing the entire microtubule array within severed melanophore arms that are devoid of pigment. (a) Low magnification phase-contrast image of the severed arms stained with tubulin antibodies as pictured in b. Black arrows indicate sites of cuts. The large bulbous region of each arm contains microtubules, some of which are random in disposition. Microtubules do become more ordered upon entry into peripheral extensions of various sizes. White arrows mark the initiation of microtubule bundle formation within the arm centers. Bar, 20 μm.

first being the microtubules themselves. One can imagine that the final position of the new center is influenced by a generated force that is dependent upon both the number and length of microtubules on either side of the migrating center. Immediately after severing, most of the microtubules, and

whatever forces they generate, would reside on the distal side of the new center (see Fig. 1, $a'-d'$). Over time, the proximal microtubules may grow and elongate, while the distal microtubules disassemble or shorten in an attempt to create an equilibrium of microtubule-propagated traction forces at the arm centroid. There is convincing evidence that such a situation exists at the metaphase plate during meiosis (Hays et al., 1982). Because many of the melanophore arms we have studied exhibit gross imbalances in the total amount of tubulin polymer found on either side of their newly formed center (Figs. 2, $a-f$, and 6 a'), it is likely that other cytoskeletal constituents are involved. An alternative model would implicate the action of a dynamic gel-like lattice-work which has been described to fill the entire cytoplasmic compartment (Buckley and Porter, 1975; Porter et al., 1983). Such a cytoskeletal configuration within each arm of a melanophore could generate and transduce pulling forces upon the cell center. When the continuity of the gel within an arm is broken by severing, it is placed in a thermodynamically unfavorable state and responds by contracting isotropically into the arm middle. This contraction would be represented by the formation and subsequent migration of a new functional center into the arm centroid. The electron micrographs in Fig. 5 support this model by showing a dramatic difference in the distribution of cytomatrix within an intact arm (Fig. 5, a and b) as opposed to a severed arm (Fig. 5, c and d). From these images, it appears as if the matrix and its associated organelles, once spread evenly throughout the intact arm, have withdrawn from the peripheral extensions to accumulate into the arm center after severing.

Are Microtubule-nucleating Elements Ubiquitous?

The fact that the reorganizational process within severed arms is indifferent to a change in the distribution of pigment or the absence of pigment granules altogether (Table III) indicates that other nongranule-associated components are responsible. Thus, it seems likely that peripheral growth and organization of noncentrosomal microtubules occurs in other types of cells and is not restricted to melanophores. It has been shown that cytoplasts of Haemanthus endosperm cells, after the enucleation process, initially form "microtubule converging centers" which are eventually transformed into spirals or chromosome-free pseudospindles (Bajer and Molè-Bajer, 1986). It is now known that amputated neurites regrow a population of newly formed microtubules outward from their centers (Bass and Heidemann, 1986; Bass et al., 1987). These nascent microtubules are believed to extend from a stable population of microtubule fragments found within the neurite (Heidemann et al., 1984), although such regrowth may instead represent the formation of a new center within the neurite, similar to the reorganization process we observe in melanophore arms. This seems most probable, particularly in view of the recent evidence implicating

Figure 5. Cytoplasmic matrix accumulates at the center of a melanophore arm after severing. High voltage electron micrographs of melanophore arms emptied of pigment, either left attached to the mother cell (a and b) or severed (c and d), then fixed, and critical point dried. In the intact arm (a and b), the distribution of matrix material is spread evenly throughout the arm cytoplasm as a whole. In severed arms, however (c and d), the matrix appears to have withdrawn from peripheral extensions (*) to gather or cluster (arrows) into the central region of the arm. Boxes show regions of higher magnification. M, mitochondria. Mt, microtubules. Bars: (a) 10 μm; (b) 5 μm; (c and d) 2 μm.
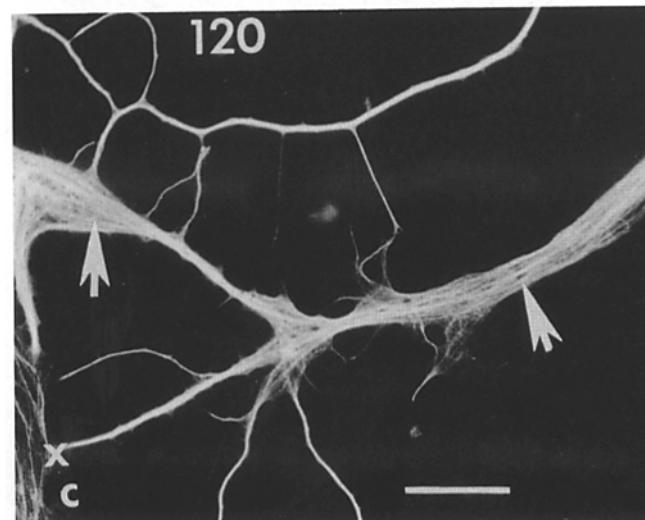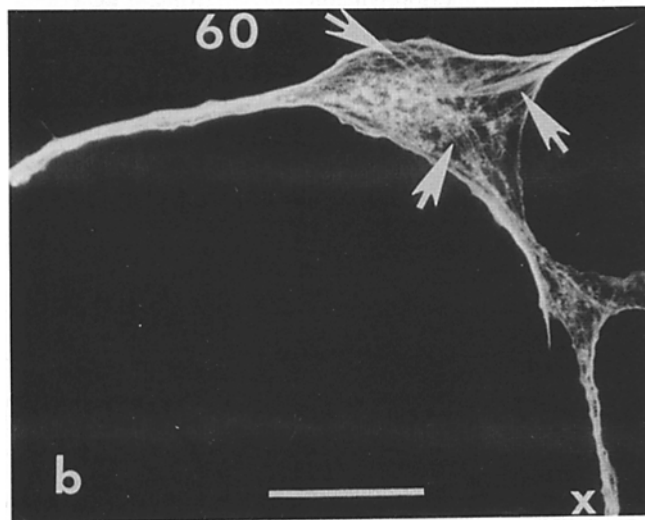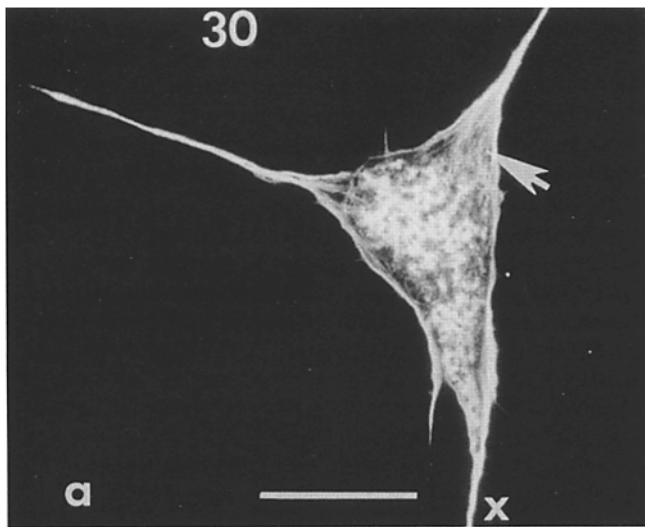
Cell		CW microtubules	CCW microtubules	CW	CCW
				%	%
A	Proximal	5	41	11	89
	Distal	80	15	84	16
B	Proximal	3	29	9	91
	Distal	58	16	78	22
C	Proximal	20	105	16	84
	Distal	NC	NC	–	–
D	Control arm*	12	45	21	79

Polarities of microtubules from severed arms (not including cell D) after a 4-h recovery from cold and nocodazole (see Materials and Methods).
* Not severed before drug and cold treatment.

microtubule assembly throughout the length of elongating neurites (Lim, S. S., personal communication) and in axonal growth cones (Bamburg et al., 1986).

Although severed arms devoid of pigment facilitated our viewing of their centers, we did not observe any specific morphological site from which microtubules extend either by electron microscopy (Figs. 4 and 5) or immunofluorescent microscopy (Fig. 6). Instead, we consistently found that the microtubules within the centroid area are disoriented and may remain so until they appear in the peripheral arm extensions. Once within the extensions, the microtubule population becomes ordered, properly oriented, and closely associated with the cortices to form the peripheral "cage" found in intact arms. This cortical association is also seen within severed arms recovering from cold and nocodazole treatment (Fig. 7). Thus, from these morphological observations, it appears that growth of microtubules within severed arms is not from a single nucleating structure but from constituents distributed throughout the arm center. This is not surprising if one recalls that the dense-staining bodies of the centrosome are not membrane enclosed but appear continuous with the surrounding cytoplasmic matrix. Indeed, we have recently found that the dense bodies of the elaborate centrosome within intact melanophores may extend up to 20 μm from the centrioles before they appear to dissipate (McNiven, M. A., and K. R. Porter, manuscript in preparation). Whether or not the peripheral constituents remain distributed throughout the arms or become localized within the arm center after severing has not been determined because staining of the arms with three different antibodies directed against centrosomal antigens has proven uninformative.

The notion that microtubule organization is controlled by factors distributed throughout the unit cytoplast of a cell, while unconventional, is not totally unique and gains support

Figure 7. Severed arms reassemble well-ordered microtubule bundles after treatment with cold and nocodazole. Fluorescent micrographs of severed arms, emptied of pigment and treated with nocodazole and cold, and then allowed to recover in drug-free media for 30, 60, and 120 min, respectively. After a 30-min recovery (a), some microtubules can be seen situated at the base of a small arm extension (arrow). Much of the remaining depolymerized tubulin appears as a bright halo around, or inside, spherical vesicles situated at the arm center. By 90 min (b), more microtubules can be seen reaching from the arm center (arrows). Fluorescence from depolymerized tubulin, while still present, is reduced in intensity. (c) By 2 h in drug-free media, most of the tubulin has reassembled into properly polarized, fully functional microtubule bundles (arrows). x, site of cut. Bars, 10 μm.

from previous studies of microtubules in many different cell types. For example, microtubules not associated with obvious nucleating structures have been observed in the marginal band of avian erythrocytes (Miller and Solomon, 1984; Swan and Solomon, 1984; Murphy et al., 1986), the unicellular algae *Distigma* (Murray, 1983, 1984) and in the neurons of the nematode *Caenorhabditis* (Chalfie and Thomson, 1979). From these examples and the new observations presented here, it is likely that the nucleation, disposition, and orientation of cytoplasmic microtubules are regulated by both centrosomal and noncentrosomal peripheral cytoplasmic sites. The elucidation of these factors and their structural interaction with other cytomatrix components will no doubt lend insight into the cytoskeletal organization of neurons and other cell types.

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