# MICROTUBULE SYSTEM OF ISOLATED FISH MELANOPHORES AS REVEALED BY IMMUNOFLUORESCENCE MICROSCOPY

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ABSTRACT

The microtubule system of melanophores of the angelfish, *Pterophyllum scalare*, has been studied using antibodies prepared against purified porcine brain tubulin in indirect immunofluorescence microscopy. Melanophores were freed from the surrounding tissue components of isolated scales by mild enzymatic digestion and then allowed to settle on a glass cover slip. In both the dispersed and the aggregated states large numbers of fluorescent fibers are seen. The number and the astral arrangement of these fibers, which run from the central region to the periphery of the cell, are striking. The system of fluorescent fibers is replaced by diffuse fluorescence of moderate intensity after cold treatment, but is restored after rewarming the cells. Differences in the immunofluorescence profiles between cells with dispersed and aggregated pigment are discussed in relation to electron microscopic data available for this system.

KEY WORDS fish melanophores · microtubules · tubulin antibodies · immunofluorescence microscopy

Microtubules constitute the major fibrous component of fish chromatophores and are especially abundant in cells which exhibit rapid translocation of pigment granules. In the discoidal or star-shaped chromatophores of several species of fish, microtubules extend along the long axis of the cell processes (1, 4, 13). Because their course also parallels the direction of pigment granule movement, and because antimicrotubular agents reduce or abolish these movements (5, 14), microtubules are thought to be involved in the processes of pigment aggregation and dispersion, although the precise mechanism of this displacement remains unknown.

Detailed knowledge of the distribution of microtubules within these cells is a prerequisite for a consideration of their function. Previous studies attempted to obtain insight into the three-dimensional arrangement of the microtubular system of angelfish melanophores; serial transverse sections were used in an electron microscopic analysis directed to tracing all microtubules within certain segments of the melanophore *in situ* (i.e., as part of the fish scale). The major conclusions from this work were that almost all microtubules originate in the cell center, and that the overwhelming majority of the microtubules can be traced over considerable distances (up to 10 $\mu$m, corresponding to 20–40% of the cell radius).

Recently, immunofluorescence microscopy used with antibodies against tubulin has proved to be a valuable tool for a fast and simple visualization of tubulin-containing cell structures including cytoplasmic microtubules (2, 3, 9, 15, 16). The main advantage of the procedure is the overview it provides of the cytoplasmic organiza-

tion of microtubules within a cell. Studies on tissue culture cells have suggested that microtubules may be followed for long distances. Our original efforts to study the microtubular system of melanophores by applying tubulin antibodies to melanophores *in situ* gave unsatisfactory results. Therefore, melanophores were freed from the surrounding skin tissue components and allowed to settle on a glass cover slip. This report describes the visualization of the microtubular apparatus of isolated fish melanophores under different conditions, using the fluorescent antibody technique.

## MATERIALS AND METHODS

### *Preparation of Isolated Melanophores*

Scales of the angelfish, *Pterophyllum scalare*, were removed with tweezers and incubated in Ringer's solution containing 0.5–1 mg/ml collagenase and 0.1–0.2 mg/ml hyaluronidase for periods of up to 1 h (see also reference 7). Melanophores were then freed from the surrounding tissue components by a gentle stream of water and allowed to attach to glass cover slips in a 4:1 mixture of Ringer's solution and amphibian culture medium (Grand Island Biological Co., Grand Island, N. Y.) for 3–16 h. Aggregation of pigment granules within the cells was induced by addition of $10^{-4}$ M epinephrine to the medium, and redispersion by addition of $10^{-4}$ M atropine.

### *Immunofluorescence Microscopy*

The procedures used are similar to those used for tissue culture cells and have been described in detail (15). Briefly, the melanophores were fixed by incubation in 3.7% formaldehyde in phosphate-buffered saline (PBS) and then incubated with cold methanol for 6 min. The cover slips were then rinsed in PBS at room temperature, and the antitubulin antibody was applied. Incubation with both the first and second antibodies was carried out for 45 min at 37°C and the cover slips were washed well after each antibody.

The tubulin antibody was made against porcine tubulin free of associated proteins and was made monospecific by passage over tubulin coupled to Sepharose 4B (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.) (17). The second antibody was fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit γ-globulin from Yeda (Research and Development Co., Ltd., Rehovoth, Israel).

## RESULTS

An analysis of isolated melanophores, including quantitative data on the microtubular apparatus under different physiological conditions, will be given elsewhere.[1] In brief, melanophores subjected to the culture conditions described above tend to lose their highly arborized outlines (Fig. 1 *a*), presumably by lateral fusion of the membranes of cell processes. The single pigment granules of the cells usually show moderate centrifugal and centripetal movements. Upon addition of $10^{-4}$ M epinephrine, the melanosomes migrate rapidly towards the central area of the cell and form a spherical pigment mass (Fig. 1 *b*), leaving the cell processes free of melanosomes and unchanged in outline. Rapid redispersion is induced when $10^{-4}$ M atropine is added to the medium. The velocities of aggregation and dispersion of the melanosomes in the isolated melanophores are similar to those of melanosomes in cells *in situ* (2 μm/s for aggregation, 0.5 μm/s for dispersion).

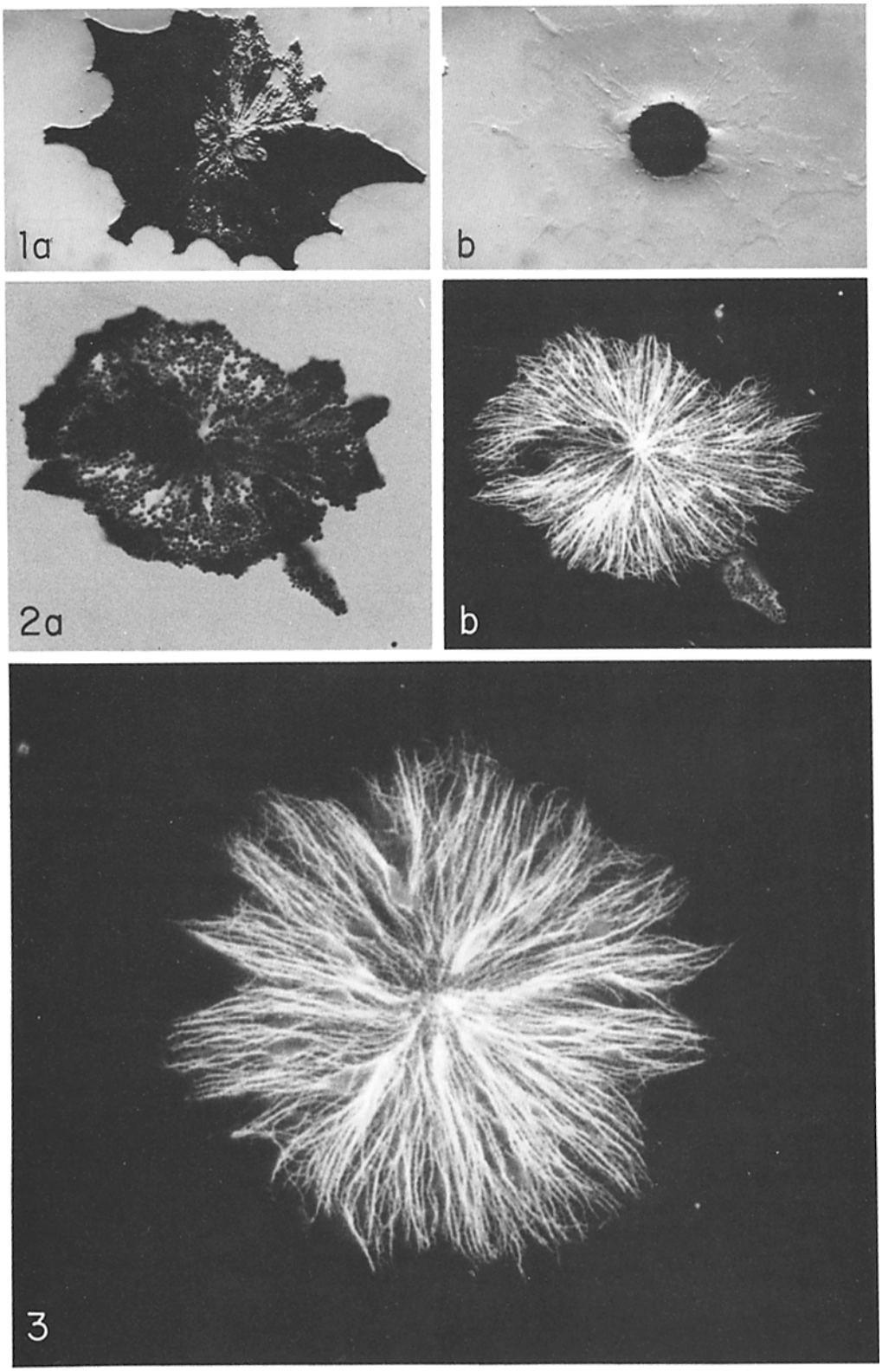
Preparations of cells with pigment dispersed (Fig. 2 *a*) reveal an elaborate system of fluorescent fibers with a strikingly radial arrangement when examined by immunofluorescence microscopy with monospecific tubulin antibody (Figs. 2 *b*, 3). Many of the fibers appear to traverse the cytoplasm, running from the central region of the cell to the outer margin of the cell where they seem to end abruptly, giving the impression of a star with numerous astral rays. The thickness (and also the brightness) of individual fibers varies, indicating that the number of microtubules constituting a fluorescent fiber may also be variable.

[1] Schliwa, M., and U. Euteneuer. Structure and physiology of isolated fish melanophores. Manuscript in preparation.

FIGURE 1 *a*, *b* Nomarski interference contrast micrographs of a living isolated melanophore attached to a glass cover slip. (*a*) dispersed state; (*b*) same cell 2 min after addition of $10^{-4}$ M epinephrine. × 450.

FIGURE 2 *a*, *b* Melanophore with pigment dispersed subjected to the indirect immunofluorescence procedure with antitubulin antibody. (*a*) phase-contrast; (*b*) same cell in fluorescence microscopy. × 1,100.

FIGURE 3 Immunofluorescence of cell with pigment dispersed. Note the striking array of astral fibers. × 1,450.


1a
b
2a
b
3

In isolated cells in which the pigment granules have been induced to aggregate in the cell center (Fig. 1*b*), the melanosome-free cytoplasm displays a multitude of fluorescent fibers arranged like those seen in cells with pigment dispersed (Fig. 4). Although hard to resolve because of their high density, most of the fibers appear to be more delicate. Grouping in bundles is not so apparent as in cells with dispersed pigment. In the region of the concentrated pigment mass, only a few fibers can be seen.

Electron microscope studies show that in angelfish melanophores microtubules are arranged predominantly around the periphery of cell processes *in situ* (12) or at the upper and lower membranes in vitro.[1] The black pigment granules are found between the upper and lower sets of microtubules. Thus, because the granules themselves are opaque to ultraviolet light, only the set of microtubules on the lower side of the cells, i.e. the cover slip side is revealed in Figs. 2 and 3. To demonstrate the upper and lower sets of microtubules in a single cell, cells were mounted between two cover slips and photographed from both sides. Because in cells of normal size it is hard to photograph the upper surface in a single focal plane as this surface is wavy and is raised in the cell center, the upper set of microtubules is difficult to document. Thus, to demonstrate the "flip-flop" technique, a small cell was selected. The pair of photographs in Fig. 5 have different microtubular profiles and show that two different sets of microtubules are indeed present. The absorbance of pigment granules is so effective that one set of microtubules can be bleached in the fluorescence microscope without affecting the other set.

The central region of the cell is usually covered in both the aggregated and dispersed states by melanosomes, on both the upper and lower sides,[1] so that the cell center can not be seen in immunofluorescence microscopy. Occasionally, melanophores in which the pigment granules are extremely well dispersed, such as the slightly asymmetric cells shown in Fig. 6*a* and *b*, can be found. These cells show a brightly fluorescent zone from which fibers radiate into the cytoplasm. This fluorescent zone most probably corresponds to the region of the central apparatus observed with the electron microscope (11).[2]

Cytoplasmic microtubules are known to depolymerize at low temperatures (see reference 8), and electron microscope studies show that the microtubules of melanophores also disappear if the cells are subjected to cold treatment (5). Isolated melanophores kept at 0° to −2°C for 30 min show a complete disappearance of the fibrous microtubular elements, in both the dispersed and aggregated states, when assayed by immunofluorescence microscopy (Fig. 7*a*, *b*). Instead, the cells display a diffuse fluorescence of moderate intensity which is higher than background. In cells with pigment aggregated, there is an increased intensity around the pigment mass (Fig. 7*b*). If melanophores are allowed to recover from cold treatment for 10 min at room temperature, a radial array of microtubular elements quite similar to that in untreated cells is observed (Fig. 8).

## DISCUSSION

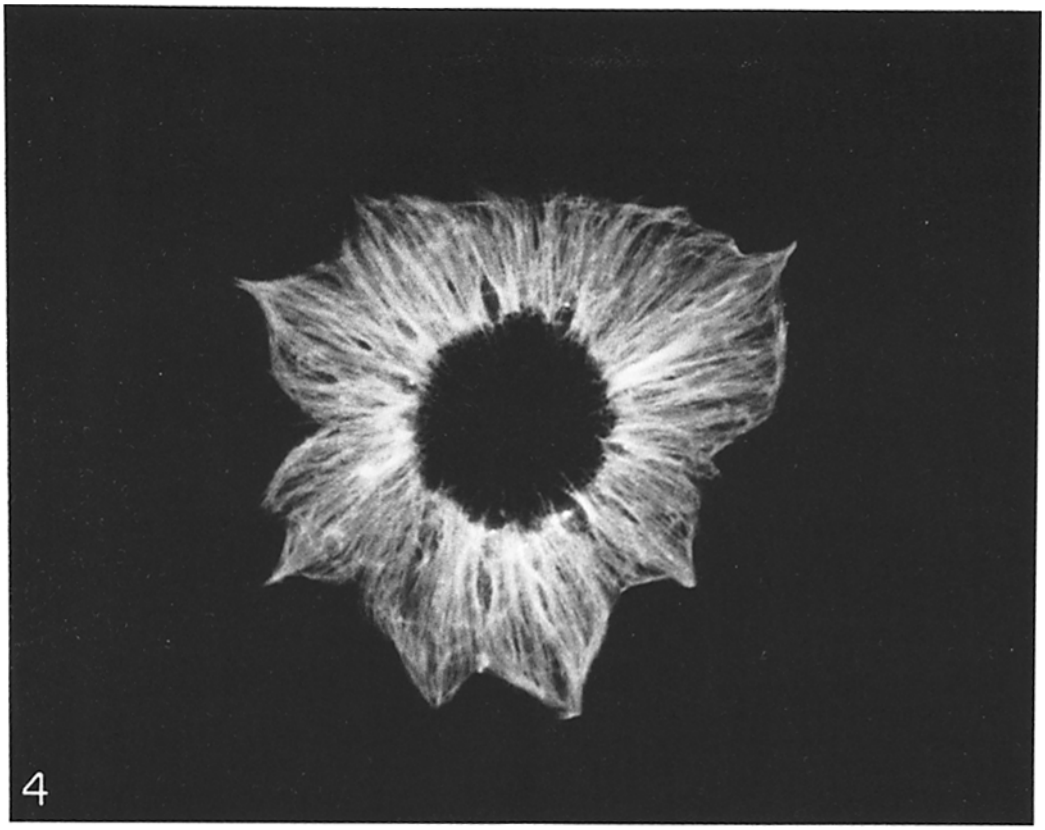
We have been able to characterize the microtubular system of isolated melanophores by using an antibody prepared against tubulin from porcine brain in immunofluorescence microscopy. Fluorescent fibers radiate from a brightly fluorescent region in the cell center and run to the periphery of the cell, maintaining a straight course. Here, they apparently terminate roughly at right angles to the cell margin. Individual fibers can be traced for long distances. Similar characteristics of microtubules in tissue culture cells have been documented (2, 9, 16), particularly during respreading (10). The fluorescent images of melanophores

[2] Schliwa, M. The microtubular apparatus of melanophores. Three-dimensional organization. Submitted for publication.

FIGURE 4 Immunofluorescence of cell with pigment aggregated. Note that the fibers are again radially arranged and seem thinner than in Fig. 3. × 1,450.

FIGURE 5 *a*, *b* A small melanophore with pigment dispersed in immunofluorescence microscopy, using the flip-flop technique, photographed to show lower (*a*) and upper (*b*) sides of the cell. × 980.

FIGURE 6 *a*, *b* Two different cells with extremely well-dispersed pigment granules showing the central cell region in immunofluorescence microscopy. These micrographs have been purposely overexposed to document the organization of the brightly fluorescent central area of the cells. × 1,620.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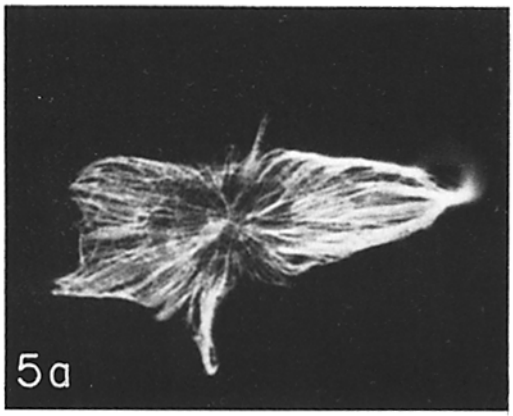
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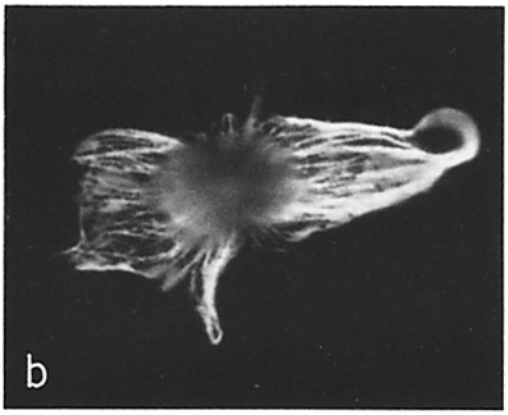
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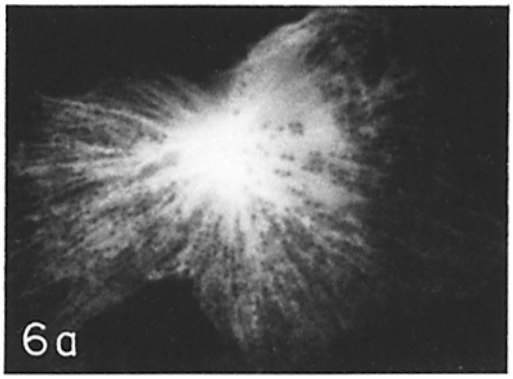
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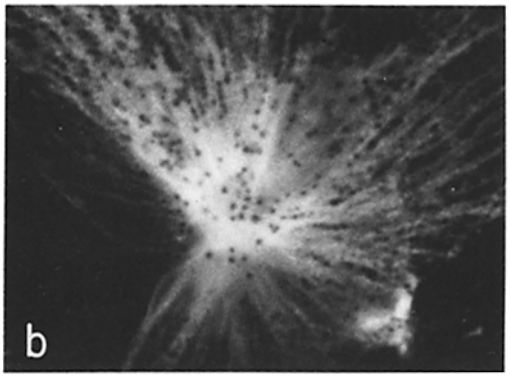
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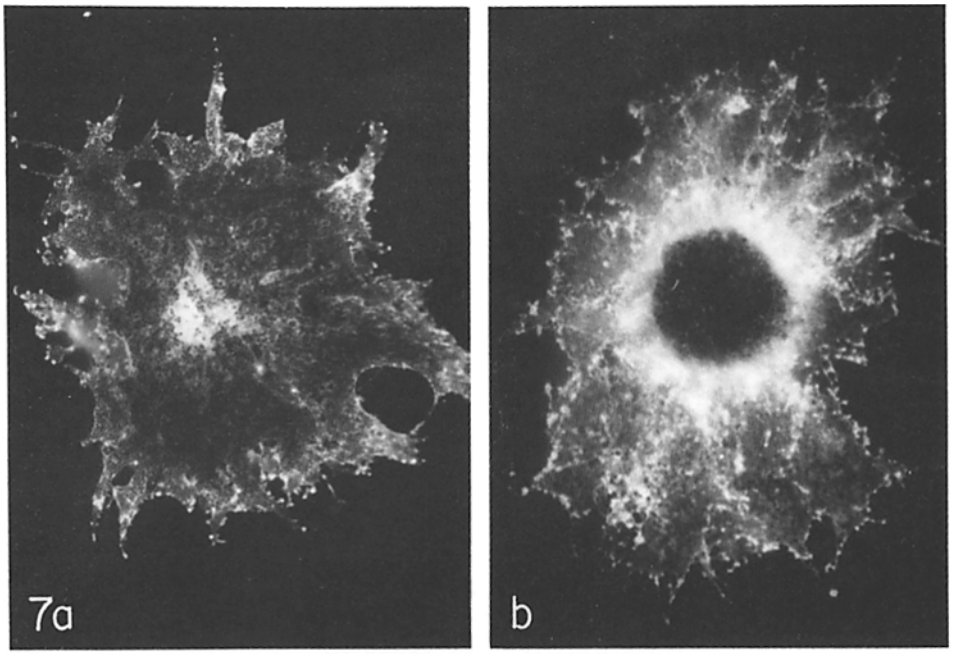


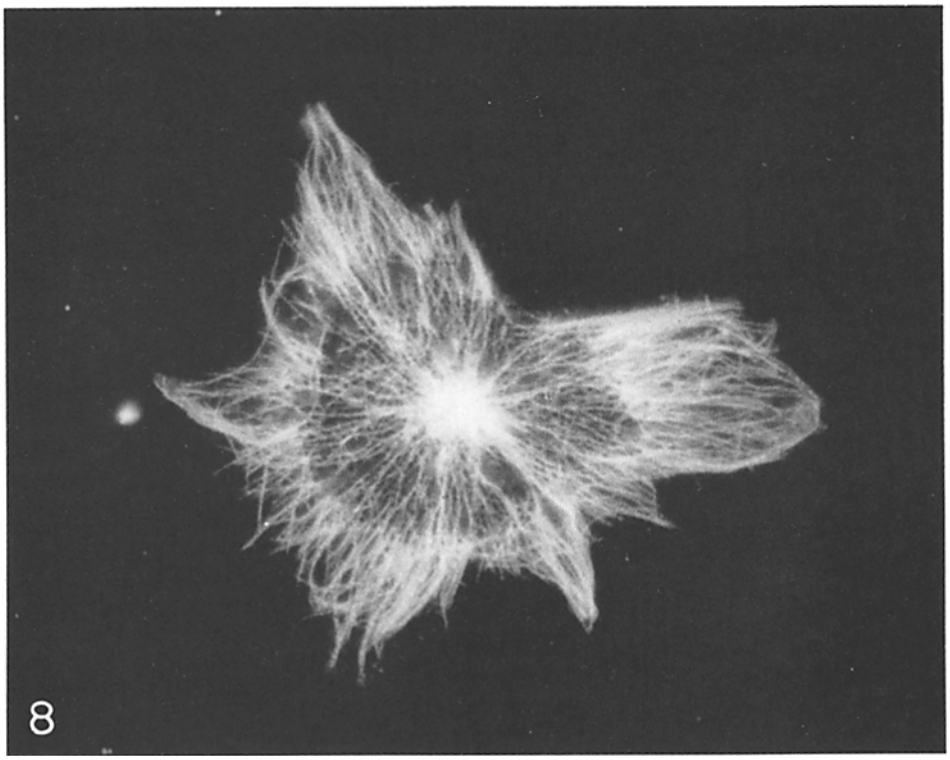


FIGURE 7 *a*, *b* Cells shifted from 0° to −2°C for 30 min and then treated with tubulin antibody and viewed in immunofluorescence microscopy. (*a*) cell with dispersed pigment, (*b*) cell with aggregated pigment. Note the loss of fluorescent fibers and the diffuse moderate background fluorescence. × 700.

FIGURE 8 A cell kept at 0° to −2°C for 30 min and then maintained for 10 min at room temperature. Note the recovery of the radial arrangement of abundant fluorescent fibers. × 950.

with pigment aggregated and dispersed, respectively, differ slightly with respect to the thickness, brightness, and concentration of the fluorescent fibers. The identification of the fluorescent fibers as microtubules includes the use of monospecific antibody against tubulin (2, 9, 17), the fact that this antibody decorates cytoplasmic microtubules and other tubulin-containing structures in tissue culture cells (2, 3, 9, 15, 16) and the demonstration in the melanophore, and in other systems (2, 3, 9, 16, 17), that they are sensitive to treatments known to depolymerize cytoplasmic microtubules.

The microtubular apparatus of melanophores *in situ* has already been studied by electron microscopy (12),[2] and analyses of the microtubule system have recently been extended to isolated melanophores.[1] Microtubules radiate from the central apparatus and, at some distance from the center, associate with the upper and lower cell membranes. Because isolated melanophores are spread flat on the cover slip, it is only the lower set of microtubules that is observed by immunofluorescence microscopy of melanophores in the dispersed state unless the flip-flop technique is applied. With respect to the general organization of microtubules, the results obtained with immunofluorescence techniques agree with the electron microscope studies. The observation of slightly thicker fluorescent fibers in cells with pigment dispersed (Fig. 3) compared with fibers in cells with aggregated granules (Fig. 4) is also in agreement with ultrastructural analyses of the microtubule disposition in the two different stages, and it may be that at least some of the very thin delicate fluorescent fibers seen in cells with aggregated pigment correspond to individual microtubules. Because of this general agreement between the impressions obtained by immunofluorescence microscopy and electron microscopy, one is tempted to ask whether fluorescence microscopy can enable us to estimate the change in total number of microtubules between the two stages. This change has previously been estimated to be a loss of ~50% in the total microtubule number upon aggregation of the pigment (13). Such a predicted change could ideally be found by fluorescence microscopy, if certain difficulties with this technique, typified by angelfish melanophores, could be circumvented. The first of these difficulties is the enormous number of microtubules present (1,500–2,500) radiating from the cell center in the dispersed state and their spatial closeness (estimate in the cortex of the cell processes, 30–40 nm [12]).[1] Thus, a situation very similar to that in the mitotic spindle is present; the abundant microtubules are so close together that the impression is given of thick fibers rather than individual tubules because of the lack of resolution. Second, in cells with pigment dispersed, usually one set of microtubules (the lower one) is accessible to fluorescence analysis short of extensive flip-flop preparations, and even then microtubules among the melanosomes are obscured by the pigment granules. Third, it has not been established how the very effective light-absorbing pigment (6) influences the absorption and/or emission of the fluorescent dye. Parallel electron microscopic analysis of melanophores from other fish species in which the microtubules are less crowded might allow the assessment of the importance of the first point, which we consider the most serious limitation for quantitative analyses of microtubules in the two stages of pigment distribution.

Our studies show that the expression of melanophore microtubules is sensitive to low temperature as previously shown for many other cells (8). The expression of fluorescent fibers is abolished in both the dispersed and aggregated states, and is replaced by a diffuse and unstructured fluorescence. Recovery from this treatment by warming restores the complex astral fibers system. These results indicate that immunofluorescence microscopy may allow us to rapidly screen the kinetics of depolymerization and repolymerization of microtubules under different conditions, such as cold treatment or incubation with drugs affecting mitosis.

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## REFERENCES

1. Bikle, D., L. G. Tilney, and K. R. Porter. 1966. Microtubules and pigment migration in the melanophores of *Fundulus heteroclitus* L. *Protoplasma.* **61**:322–345.
2. Brinkley, B. R., G. M. Fuller, and D. P. Highfield. 1975. Cytoplasmic microtubules in normal and transformed cells in culture: analysis by tubulin antibody immunofluorescence. *Proc. Natl. Acad. Sci. U. S. A.* **72**:4981–4985.
3. Frankel, F. R. 1976. Organization and energy dependent growth of microtubules in cells. *Proc. Natl. Acad. Sci. U. S. A.* **73**:2798–2802.
4. Junqueira, L. C., E. Raker, and K. R. Porter. 1974. Studies on pigment migration in the melan-

ophores of the teleost *Fundulus heteroclitus* (L.). *Arch. Histol. Jpn.* **36:**339–366.

5. MURPHY, D. B., and L. G. TILNEY. 1974. The role of microtubules in the movement of pigment granules in teleost melanophores. *J. Cell Biol.* **61:**757–779.
6. NICOLAUS, R. A. 1968. Melanins. Hermann, Paris. 310 pp.
7. OBIKA, M. 1976. Pigment migration in isolated fish melanophores. *Annot. Zool. Jpn.* **49:**157–163.
8. OLMSTED, J. M., and G. G. BORISY. 1973. Microtubules. *Annu. Rev. Biochem.* **42:**507–541.
9. OSBORN, M., and K. WEBER. 1976. Cytoplasmic microtubules in tissue culture cells appear to grow from an organizing structure towards the plasma membrane. *Proc. Natl. Acad. Sci. U. S. A.* **73:**867–871.
10. OSBORN, M., and K. WEBER. 1976. Tubulin specific antibody and the expression of microtubules in 3T3 cells after attachment to a substratum. *Exp. Cell Res.* **103:**331–340.
11. PORTER, K. R. 1973. Microtubules in intracellular locomotion. *Ciba Found. Symp.* **14:**149–166.
12. SCHLIWA, M. 1975. Microtubule distribution and melanosome movements in fish melanophores. *In* Microtubules and Microtubule Inhibitors. M. Borgers and M. de Brabander, editors. North-Holland Publishing Company, Amsterdam. 215–228.
13. SCHLIWA, M., and J. BEREITER-HAHN. 1973. Pigment movements in fish melanophores: morphological and physiological studies. II. Cell shape and microtubules. *Z. Zellforsch. Mikrosk. Anat.* **147:**107–125.
14. SCHLIWA, M., and J. BEREITER-HAHN. 1973. Pigment movements in fish melanophores: morphological and physiological studies. III. The effects of colchicine and vinblastine. *Z. Zellforsch. Mikrosk. Anat.* **147:**127–147.
15. WEBER, K., T. BIBRING, and M. OSBORN. 1975. Specific visualization of tubulin containing structures in tissue culture cells by immunofluorescence. *Exp. Cell Res.* **95:**111–120.
16. WEBER, K., R. E. POLLACK, and T. BIBRING. 1975. Antibody against tubulin: the specific visualization of cytoplasmic microtubules in tissue culture cells. *Proc. Natl. Acad. Sci. U. S. A.* **72:**459–463.
17. WEBER, K., J. WEHLAND, and W. HERZOG. 1976. Griseofulvin interacts with microtubules both in vivo and in vitro. *J. Mol. Biol.* **102:**817–829.