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Visualization of microtubules of cells *in situ* by indirect immunofluorescence

(microtubule organizing centers/cytoskeleton/chromatophores/scleroblasts/orthogonally arranged collagen)

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ABSTRACT **Microtubule staining patterns can be visualized within cells *in situ* on the surface of fish scales from the squirrel fish, *Holocentrus ascensionis*, and the common goldfish, *Carassius auratus*, after incubation with antibodies to sea urchin tubulin and fluorescein-labeled goat antibodies to rabbit immunoglobulin G. Chromatophores *in situ* from both species reveal a radial microtubule framework that orients the alignment of pigment granules. Innervating fibers of erythrophores on the *H. ascensionis* scale can also be observed. *In situ*, pseudo-epithelial cells called scleroblasts show microtubule patterns with a remarkable degree of similarity within a selected region. Over 90% of the cells have a microtubule framework that is nearly superimposable from cell to adjacent cell. The microtubules in scleroblasts are few and form a simple radial framework with a localized microtubule organizing center (MTOC). Microtubules in scleroblasts *in vitro* emanate from localized MTOCs but are much less radially organized than *in situ*. Scleroblasts *in situ* on the scale of *C. auratus* show microtubules that curve abruptly into coalignment with phase striations on the fibrillary plate. The phase striations arise from the orthogonal plies of collagen in intimate association with the scleroblasts. The role of microtubules in scleroblasts may thus be to provide orientation for collagen fibrillogenesis, analogous to their role in orientation of cellulose fibers in plants. That cells *in situ* exhibit highly related and coordinated microtubule staining patterns reaffirms that the cytoskeleton plays an important role in the organization of differentiated tissues.**

The distribution of cytoskeletal proteins within nonmuscle cells has in recent years been largely elucidated by using fluorescently labeled antibodies. By this method it has been possible to visualize actin (1) myosin (2, 3), microtubules (4–6), and other proteins that are known to play a role in cell motility (7, 8). Most, if not all, of these studies have utilized tissue culture cells. In order to evaluate better the significance of observations on the cytoskeleton from cells *in vitro*, we have initially chosen to study the microtubule patterns that can be exhibited from cells *in situ* by immunofluorescence. The opportunity to visualize microtubules by this method in cells *in situ* is limited to cells in optically transparent tissues. Thus, we have found that the fish scale is well suited for the examination by phase contrast and epifluorescence microscopy because the cells on the scale are thinly spread on the surface of a nearly transparent substrate. In the present study we have undertaken a comparison of the microtubule staining patterns of chromatophores and scleroblasts *in situ* on the scales from two teleost species: the marine squirrel fish, *Holocentrus ascensionis*, and the common freshwater goldfish, *Carassius auratus*. In addition, we have compared the microtubule staining patterns of *Holocentrus* scleroblasts *in situ* with those patterns observed from scleroblasts *in vitro*.

MATERIALS AND METHODS

Organisms. Several squirrel fish, *Holocentrus ascensionis*, were captured at the West Indies Laboratory, Fairleigh Dickinson University, St. Croix, VI, and transported to the Marine Biological Laboratory, Woods Hole, MA. The goldfish, *Carassius auratus*, were obtained from the local pet vender.

Antibodies. Vinblastine-induced tubulin paracrystals isolated from sea urchin egg were used to raise anti-tubulin antibody by procedures discussed elsewhere (6). Fluorescein-conjugated goat anti-rabbit IgG (FL-GAR) (lot 5319) was purchased from Miles-Yeda.

Treatment of Scales. Using blunt forceps, we remove scales from the fish and place them into either phosphate-buffered saline (P_i/NaCl) or marine P_i/NaCl (M-P_i/NaCl). The scales are then fixed in either 0.1 M 1,4-piperazinediethanesulfonic acid/0.1% glutaraldehyde/2% (wt/vol) paraformaldehyde/2 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid/2 mM $MgCl_2$ or 3.7% (wt/vol) formaldehyde in P_i/NaCl or M-P_i/NaCl for 15 min. They are then rinsed three times in P_i/NaCl or M-P_i/NaCl and treated with 0.2% Triton X-100 in P_i/NaCl or M-P_i/NaCl for 2 min to render the membranes permeable. After three washes in P_i/NaCl for 5 min each, the scales are incubated for 45 min at 37°C with 20 μl of the anti-tubulin antibody at a concentration between 0.5 and 0.25 mg/ml of an ammonium sulfate-precipitated IgG fraction of the immune serum. After three rinses in P_i/NaCl, the scales are incubated again at 37°C with 25 μl of 1:250 dilution of FL-GAR for 45 min. After three more washes in P_i/NaCl, the scales are immersed in a solution of 25% (vol/vol) glycerol in 50 mM Tris·HCl buffer, pH 9.0, and mounted on a microscope slide with the underside of the scale facing upwards.

Tissue Culture. Scleroblasts are dissociated from *Holocentrus* scales after incubation in Ca^{2+} and Mg^{2+}-free M-P_i/NaCl containing 0.1% collagenase and 0.15% hyaluronidase. This method, previously described for chromatophores (9), works equally well for the isolation of scleroblasts from the scale. The scleroblasts are then cultured on carbon-coated glass coverslips, fixed, and treated with antibodies in a manner similar to that used on scales in the preceding section.

Light Microscopy. All micrographs shown are taken with a Leitz Orthoplan microscope stand equipped with a Ploem vertical illuminator and a Zeiss ×63 (numerical aperture 1.4) Planapo phase-contrast objective lens. Excitation of fluorescein and the filter combinations used are described elsewhere (3).

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Abbreviations: FL-GAR, fluorescein-conjugated goat anti-rabbit IgG; P_i/NaCl, phosphate-buffered saline; M-P_i/NaCl, marine P_i/NaCl; MTOC, microtubule organizing center.

RESULTS

Immunofluorescent Staining of *C. auratus* Scales. When scales are treated with the anti-tubulin and FL-GAR antibodies, a considerable amount of diffuse background fluorescence is often observed on the scale, as seen in Fig. 1*B*. Nevertheless, radiating microtubule staining patterns can clearly be identified where scleroblasts (10) are found on the surface of the fibrillary plate of the scale (Fig. 1). A comparison of the phase-contrast images with the fluorescent images reveals that the direction of parallel phase-dense striations tends to coincide with microtubule patterns. In Fig. 1*A*, the orthogonal arrangement of two successive layers out of several orthogonal layers of collagen in the fibrillary plate can be readily appreciated. By using epifluorescence, microtubules in the scleroblasts are found to be aligned in the direction of the uppermost phase-dense striations closest to the scleroblasts. The microtubules initially emanate from the centrosphere and at various distances turn abruptly into alignment with the phase striations. Another subpopulation of microtubules radiating from the cell center appears to avoid this alignment.

Microtubules of chromatophores can also be identified *in situ* with anti-tubulin and FL-GAR antibodies. In Fig. 2*A*, the phase-contrast image of a xanthophore is partially disrupted by the phase-bright growth annulae on the opposite side of the

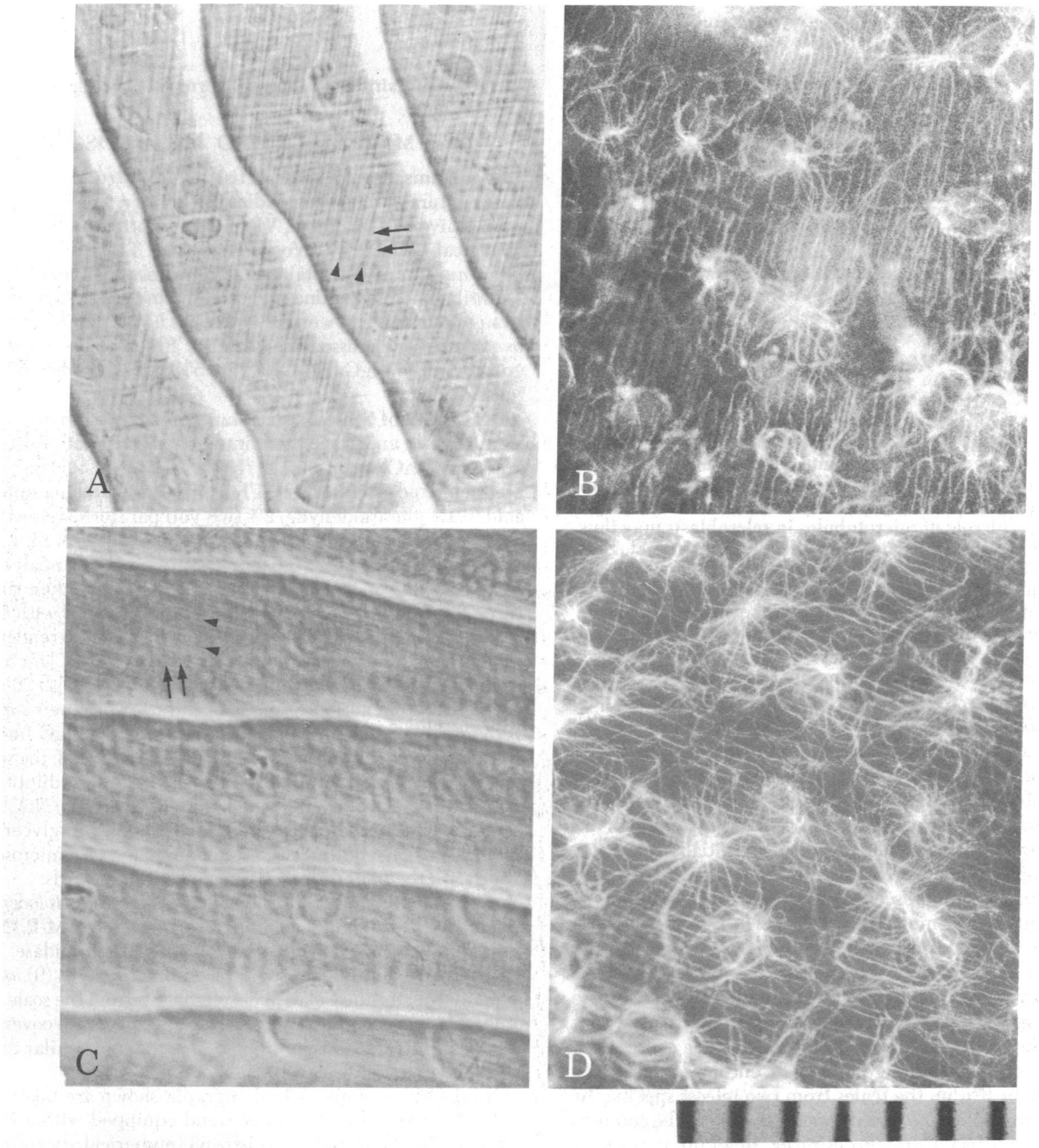

FIG. 1. (*A* and *C*) Phase-contrast image of the fibrillary plate on the undersurface of a scale from *C. auratus*. The four prominent phase stripes extending across the fields are the growth annulae on the opposite side of the scale, and two plies of the orthogonally arranged collagen fibers of the fibrillary plate are visualized as parallel phase striations (arrows and arrowheads). The upper ply of the parallel striations is in focus (arrowheads). (*B* and *D*) Epifluorescence image of *A* and *C*, respectively, of scleroblasts stained with anti-tubulin and FL-GAR antibodies. The alignment of microtubules with the phase striations is evident. Note that the microtubules emanate from localized microtubule organizing centers (MTOCs) in the center of the cell. A considerable amount of background fluorescence reduces the contrast of these preparations. Magnification scale at bottom: 1 division = 10 μm.

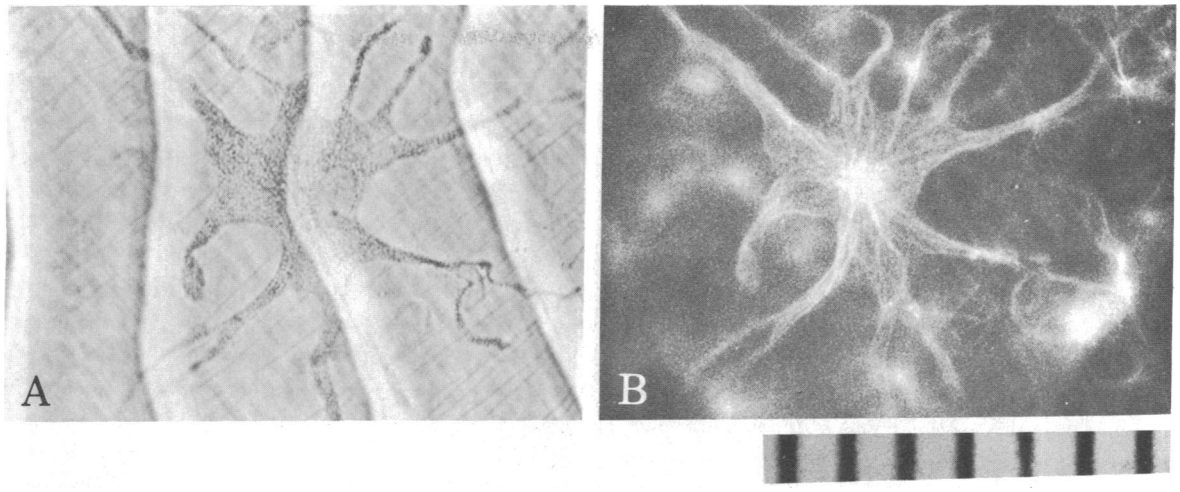

FIG. 2. (*A*) Phase-contrast image of a goldfish xanthophore *in situ*. (*B*) Epifluorescence image of xanthophore in *A*, after treatment with anti-tubulin and FL-GAR. Microtubules can be seen radiating from a highly fluorescent cell center into the dendritic processes of the xanthophore. One division = 10 μm.

scale. After examination with epifluorescence, however, (Fig. 2*B*), it is evident that the xanthophore is relatively flat, and this permits ready visualization of a radial microtubule framework.

Immunofluorescent Staining of *H. ascensionis* Scales. Microtubule staining patterns of scleroblasts on the fibrillary plate of *H. ascensionis* also demonstrate an orientation with the underlying collagen, although this alignment is much less pronounced than in images obtained from *C. auratus*. What appears to be more striking, however, is that within various regions on the scale the radial microtubule staining patterns within adjacent cells are nearly superimposable (compare Fig. 3 *A* and *B*). Indeed, nearly 90% of 210 observed scleroblasts had a microtubule framework highly related to an adjacent cell. The microtubules are few and are remarkably linear over 20 or more μm before reaching the cell perimeter. In areas on the scale

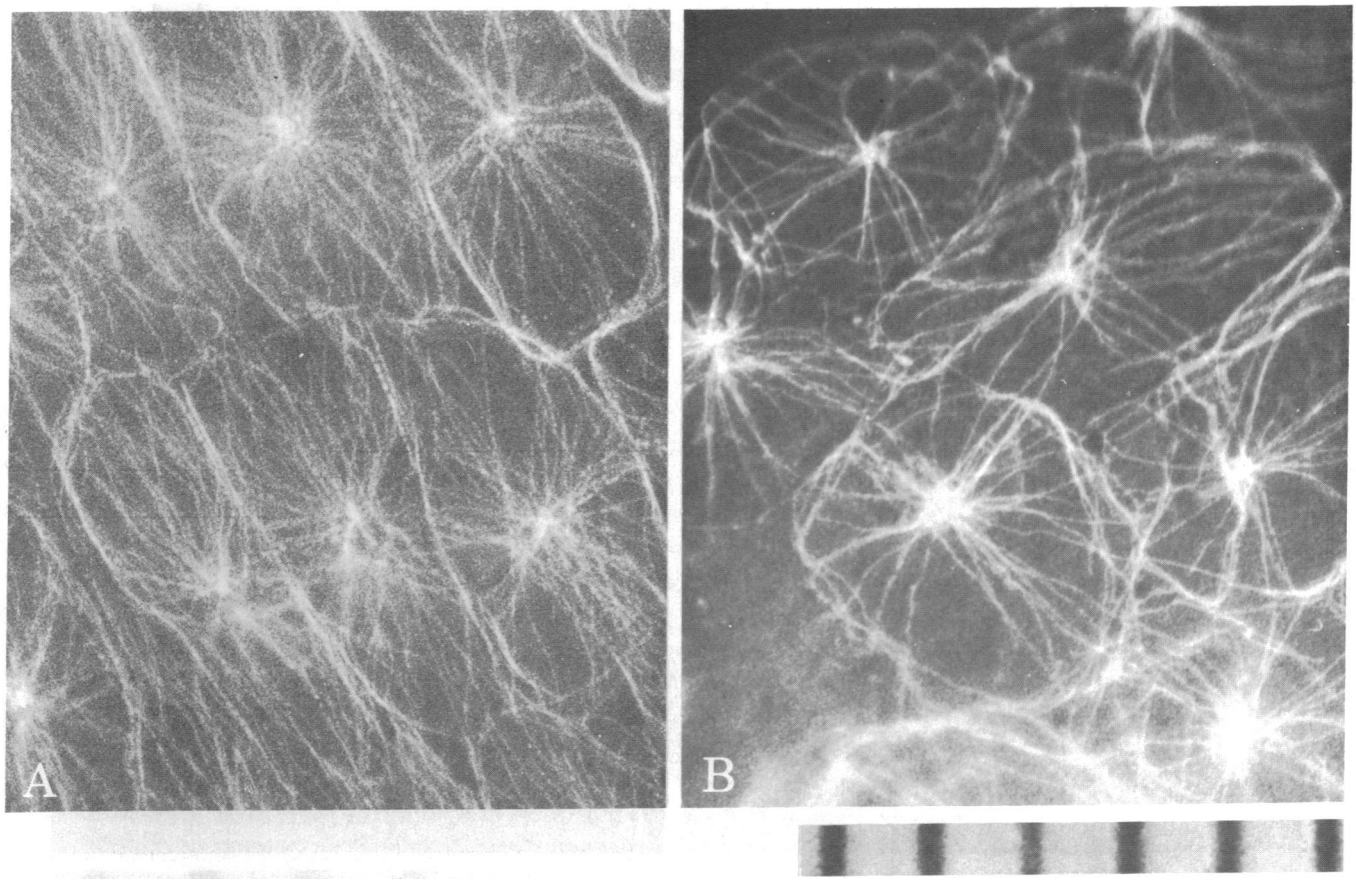

FIG. 3. (*A*) Epifluorescent image of *H. ascensionis* scleroblasts *in situ*, after treatment with anti-tubulin and FL-GAR. The microtubule staining pattern of one cell is strikingly similar to that of an adjacent cell. Few microtubules radiate from the localized MTOCs. The microtubules are remarkably linear until they reach the cell margin, where they then curve sharply and delineate the cell perimeter. (*B*) Epifluorescent image of *H. ascensionis* scleroblasts *in situ* treated as in *A* but found near the chromatophore region. In these more circular cells, fewer microtubules are found emanating from the localized MTOCs and an even greater adherence to a radial organization is maintained. One division = 10 μm.

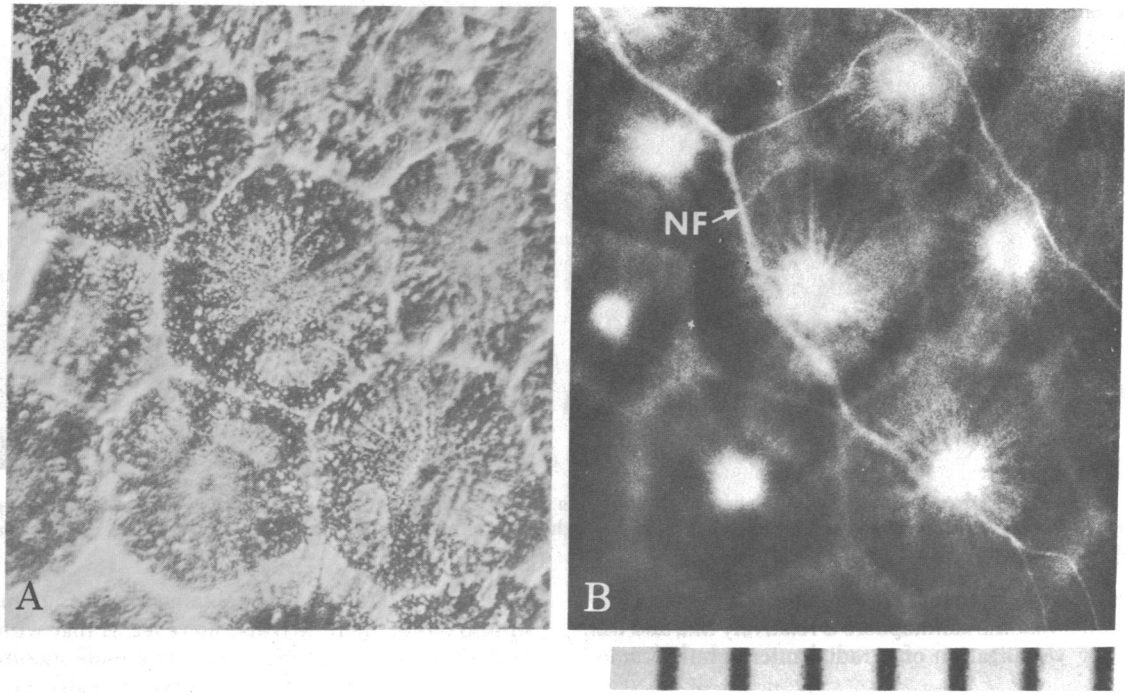

FIG. 4. (*A*) Phase-contrast image of the erythrophore region on the scale. Note the radial alignment of pigment granules and the hexagonal packing of the cells. (*B*) Epifluorescent image of the same field as in *A*. Note that the radial pattern of the microtubules emanates from a highly centralized and localized cell center. The high level of background fluorescence in this region can be appreciated by noting the pronounced quenching of the background by the pigment granules. Microtubules in nerve fibers (NF) can also be visualized in these preparations. One division = 10 μm.

adjacent to the chromatophore region, where the scleroblasts are more circular (Fig. 3*B*), there are fewer microtubules per cell than in the elongated cell region (Fig. 3*A*).

There is also an observable variation in the number and distribution of MTOCs in the scleroblasts from one region of the scale to the next (compare Fig. 3 *A* and *B*). However, in general, the MTOCs in scleroblasts are quite localized and are found in the geometric center of the cell.

The radial microtubule framework of chromatophores can also be visualized *in situ*. However, due to the background fluorescence caused by the iridophore and epithelial cell layers residing above and below the erythrophores, only the large number of microtubules per unit area in the central region can be seen (Fig. 4*B*). Nevertheless, by decreasing print exposures, the radial microtubules can be shown to extend into the cell periphery. It is interesting to note that the microtubule-containing nerve fibers are occasionally stained and are seen traversing along rows of the hexagonally arranged erythrophores (Fig. 4*B*).

Immunofluorescent Staining of *H. ascensionis* Scleroblasts *in Vitro*. Scleroblasts that are enzymatically dissociated from the scales spread well on carbon-coated coverslips after several hours in culture. The microtubules in cultured scleroblasts clearly emanate from a single and centrally located MTOC, as they do in these cells *in situ*. However, the microtubules in the cultured scleroblasts are much less linear than those observed in the radiating microtubule staining patterns of scleroblasts *in situ*. This tendency of the microtubules to undulate and curve into loops in the cultured scleroblast is illustrated in Fig. 5*B*. Although the number of microtubules is not significantly different within cultured scleroblasts and scleroblasts *in situ* on the scales, it is clear that the degree of

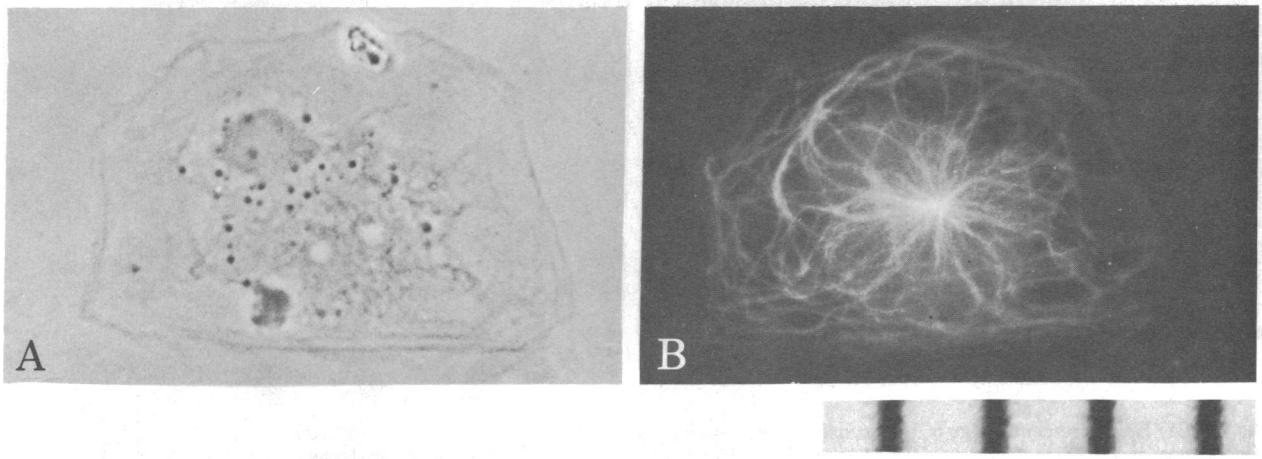

FIG. 5. (*A*) Phase-contrast image of cultured *Holocentrus* scleroblast. (*B*) Epifluorescent image of microtubule staining pattern showing a localized MTOC and emanating microtubules. Note that the linearity of microtubules *in vitro* is much less pronounced than observed in *Holocentrus* scleroblasts *in situ*. One division = 10 μm.

radial organization of the microtubules is reduced when the cell is cultured, even when the cells are in contact.

Controls for Immunofluorescent Staining. When the controls for indirect immunofluorescence are carried out, all scales exhibit a considerable degree of diffuse background staining that is not attributable to autofluorescence. In particular, simple treatment with FL-GAR alone gives rise to this background fluorescence. The nonspecific binding appears brightest on the edge of the scales and in the epidermal cell region. Preimmune serum also yields diffuse staining similar to the FL-GAR treatment alone. The only other morphological patterns observed with these staining procedures are those in the iridophore region, where the oriented cystals within these cells either do not take up the generalized staining or perhaps quench the underlying diffuse staining from below so as to produce broad dark segments within the field of diffuse fluorescence. Competition of the 1:250 dilution of FL-GAR with pretreatment of the scales with a 1:50 dilution of GAR after the initial anti-tubulin antibody incubation drastically reduces the microtubule staining patterns. Some microtubules are barely detectable in the negatives yet cannot be visualized when printed at the same exposure as the preparations that had the unlabeled GAR left out.

DISCUSSION

The current study has documented that fish scales can be used for the immunofluorescent staining of cells *in situ*. Visualization of microtubule patterns of cells *in situ* permits one to evaluate better the significance of similar patterns observed from cells in culture. We have verified that the radial and curvilinear microtubule distribution observed in scleroblasts on the under surface of the fish scale can also be seen *in vitro*. However, the *in situ* microtubule patterns exhibit a greater degree of organization and similarity between cells than that observed *in vitro*.

The orthogonal phase striations observed in this study are clearly the result of the orthogonally arranged collagen fibers in the fibrillary plate (10). Other tissues as well as the fibrillary plate exhibit orthogonally arranged collagen plies such as the basal lamella of animal epidermis (11) and that of the cornea (12). The scleroblasts, cells that reside as a pseudo-epithelium on the fibrillary plate (10), are fibroblastic in that they appear to synthesize the collagen of the plate and are not separated from the collagen fibrils by a basement membrane (ref. 10; unpublished data). The coincidence between the orientation of the radial microtubules that subsequently bend into alignment with the uppermost layer of phase striations clearly demonstrates that microtubules are aligned with the uppermost layer of collagen fibrils. It is intriguing to postulate that the microtubules may be involved in the orientation of the successive plies of collagen by orienting the fibrillogenesis of collagen, analogous to the evidence that microtubules play a role in the orientation of cellulose fibrils in plants (13, 14).

The epithelial (endothelial) 3T3 mouse cell line demonstrates a single dominant microtubule initiation site (5), whereas fibroblastic cell lines often have multiple initiation sites (15). In general, cells *in vitro* show MTOCs that are diffusely located in the centrosphere or perinuclear area, whereas scleroblasts show the MTOCs to be more discretely localized in the cell center. The MTOCs of undifferentiated neuroblastoma cells appear to aggregate as these cells are induced to differentiate (16). Thus, the scleroblasts *in situ* and *in vitro* may contain highly focalized organizing centers precisely because they are differentiated cells. Indeed, chromatophores are also highly differentiated cells, and they exhibit MTOCs that are also highly localized in culture (17, 18) as well as *in situ*.

The microtubule patterns of scleroblasts *in situ* clearly show a greater degree of radial organization than is characteristic of cultured scleroblasts and of fibroblast or epithelial cell lines observed *in vitro*. The microtubule patterns of cultured 3T3, Pt K2 (potoroo), or HeLa (human) cells (4–6) exhibit a generalized radial arrangement, but contain a greater number of microtubules and show a greater degree of complexity (more intersections, undulations, and initiation sites) than the patterns observed in the scleroblasts. This difference may be due to the fact that cultured cells are motile and are going through various phases of the cell cycle. A secondary level of greater organization not observed in cultured cells appears with the alignment of microtubules with an extracellular matrix. And the third and perhaps most striking difference is the close relatedness of the microtubule patterns of adjacent cells *in situ*, a similarity of microtubule patterns that is not evident in cultured cells (19). That microtubule patterns of scleroblasts *in situ* are nearly identical or superimposable in pairs of adjacent cells may be due to a heritable morphology reflected in cell shape (20) and distribution of cytoskeletal elements (19, 21). Thus, the microtubule frameworks that are observed in cells *in situ* are highly related, organized, and even show coordination with adjacent cells and the extracellular matrix. These observations provide further evidence that microtubules, as components of the cytoskeleton, play a significant role in a higher level of structural organization required for the morphogenesis of differentiated tissue (22).

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