

# OPTICAL DIFFRACTION AND TRANSLATIONAL REINFORCEMENT OF MICROTUBULES HAVING A PROMINENT HELICAL WALL STRUCTURE

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## INTRODUCTION

Optical diffraction patterns obtained from electron micrograph images showing crystalline or repeating units can provide confirmatory and even new information regarding substructure (7, 10, 11). Essentially, an image of a portion of an electron micrograph is used as a two-dimensional grating to produce a diffraction pattern. This pattern of spots can indicate major and minor periodicities within the subject, as well as provide the basis for measurements of subunit spacing. Although an optical bench with a laser as a coherent light source is required to obtain maximum information (11), some information can be obtained with a minimum investment by using the light microscope as a diffractometer (7). Also, information on radial and linear periodicity can be obtained by photographic means through incremental rotation or translation (6, 12, 13). In this method, an enlarged image of the specimen being tested is commonly projected on photographic printing paper, which is then rotated or translated through a series of predetermined increments. Reinforcement is obtained when the chosen increments correspond to a repeat pattern in the image.

In the present study, images of negatively stained cytoplasmic microtubules from frog lung-fluke (*Haematoloechus medioplexus*) sperm were subjected to optical diffraction and translational reinforcement. Microtubules of fluke sperm are well suited for such studies, for they are relatively stable, easily negatively stained, and have repeating transverse striations which apparently reflect an underlying helical pattern (2-4). Information obtained in this study is correlated with previous measurements and observations by the author, and a model is discussed which satisfies the available data.

## MATERIALS AND METHODS

The methods for negative staining and obtaining electron micrographs of fluke sperm are described else-

where (2, 3), although in the present study 1 N potassium hydroxide was used instead of sodium hydroxide to bring the 1% phosphotungstic acid to pH 6.8.

The system used for optical diffraction was essentially that described by Gall (7). A Zeiss WL microscope was used with a 100  $\mu$  aperture mounted below the condenser. A first-surface mirror was employed to reflect light from an Osram HBO 200 Super Pressure mercury lamp (Wild Heerbrugg, Berlin) through the aperture, above which was positioned a green interference filter (Carl Zeiss Inc., New York) transmitting a peak wavelength of 546 m $\mu$ . The mercury vapor lamp was mounted in a Wild universal lamp housing (Wild Heerbrugg Instruments, Inc., Farmingdale, N. Y.). Photographs of diffraction patterns were recorded on Polaroid Type 107/3000 speed pack film with exposures of 15 sec or less, and negatives were obtained from such prints by photocopying with Kodak Panatomic-X film.

While this manuscript was being reviewed, a laser system became available and was used to verify the diffraction patterns obtained with the mercury lamp. The beam from a helium neon laser (University Labs, Berkeley, Calif., Model L240) was directed through an objective lens, a 25  $\mu$  pinhole, and a Wollensak Raptar 162 mm lens. The same masks used to obtain patterns with the mercury lamp system were placed behind the 162 mm lens, and the diffraction patterns were recorded with a lensless camera.

Masks of microtubules to be examined were made by first photocopying positive prints of suitable micrographs on 35 mm film. A Polaroid MP-3 camera was used to obtain the necessary enlargement or reduction of the area to be diffracted, and both Kodak Panatomic-X and Contrast Process film were utilized. Generally, masks were constructed as described elsewhere (1, 7). Measurements of distances in the diffraction patterns were made possible by calibrating the system by measuring diffraction patterns of specimen screens or images of periodic structures having known spacing.

For purposes of translational reinforcement, a stereotactic device was modified by adding a Plexiglas platform to hold photographic paper in a horizontal position. Linear translations of less than 1 mm could be obtained with an incremental feed mechanism. Masks from the optical diffraction studies were

used as specimens, and the width of the microtubules to be studied was determined by referring to the original micrographs. For study, a mask was sandwiched between two clean slides, mounted in the enlarger, and its image projected on the Plexiglas platform. Double-sided tape was used to hold photographic printing paper flat upon the platform. Dimensions of the projected image were obtained, in millimeters, and converted to Ångstrom equivalents ( $x \text{ mm} = y \text{ Å}$ ). It was then a simple matter to extrapolate the incremental advances, in millimeters, necessary to move the photographic paper a distance equivalent to a predetermined number of Ångstrom units. Since a repeat of 80 Å was predicted, it was customary to test for reinforcement at intervals of 50, 80, and 110 Å. The number of incremental translations was usually 12–15, and exposure and development times for the three prints in a series were identical.

### OBSERVATIONS

Many of the masks give prominent and uncluttered diffraction patterns (Figs. 2, 5, 8, 9). The large central spot is elongate in a horizontal direction, and increasingly smaller and less intense spots may be distributed laterally along this axis; a vertical axis can be established by drawing a line perpendicular to the horizontal axis exactly through the center spot.

Fig. 1 shows the microtubular elements of a portion of a negatively stained sperm, and the usual difference between its cytoplasmic microtubules and doublet tubules is readily apparent. The microtubules retain their striated appearance and approximate dimensions in such preparations, while doublet elements become flattened to reveal the longitudinal "protofibrils" of their walls. A mask was prepared of two of the microtubules shown in Fig. 1, and its positive image is shown to the right. Diffraction patterns from these microtubules are shown in Figs. 2 and 8. A row of spots is seen above and below the horizontal axis, and the distance between each row of spots and the horizontal axis corresponds to a linear repeat distance of 80 Å. A marker line at the upper right in all photographs of diffraction patterns indicates the 80 Å "layer line." The multiple spots on the 80 Å lines indicate the presence of more than one microtubule, although all with a major periodicity of 80 Å, as confirmed by patterns obtained from the individual microtubules of such groups. Single masks were prepared of up to three adjacent microtubules, and diffraction patterns in all cases showed a row of spots along the 80 Å line. In Fig.

2, two additional spots (arrows) are displaced to the right and left of the vertical axis. These occur near the 160 Å lines, and suggest a secondary periodicity of this dimension, although this is not confirmed in the pattern (Fig. 8) obtained with laser illumination.

The same mask used to obtain Figs. 2 and 8 was tested for translational reinforcement, and Figs. 3 *a-c* show the reinforcement series obtained when the photographic paper is translated in increments equaling 50, 80, and 110 Å. Strong reinforcement is noted in Fig. 3 *b*, indicating a basic subunit repeat at linear intervals of about 80 Å.

Fig. 5 is the diffraction pattern obtained from a portion of the single microtubule shown in Fig. 4. A positive image of the mask used is displayed to the right of Fig. 5, and the mask is of the region indicated by the arrow in Fig. 4. Two spots, one less intense than the other, are on each 80 Å line; this pattern is characteristic of most of the single microtubules examined and is also evident in patterns obtained with laser illumination (Fig. 9). The more intense spots on the 80 Å line are apparently from the uppermost surface of the microtubule, where the contrast produced by the stain is greatest, while the less intense spots in the pairs are considered to be produced by the side of the microtubule resting on the specimen screen, where stain provides less contrast. The two intense spots of the pairs are on opposite sides of the vertical axis, which is compatible with a helical configuration, and the angle between a line drawn through the intense spots and the vertical axis is about  $20^\circ$ , indicating the inclination of the helix. When the microtubules shown in the mask beside Fig. 1 are individually examined, they give diffraction patterns having a helix inclination of  $15^\circ$ , and in all single microtubules giving a prominent diffraction pattern the helix is inclined  $15\text{--}20^\circ$ . An exception is noted in a pattern obtained from a microtubule pretreated with 1 M mercaptoethanol, where the inclination of the helix is about  $35^\circ$ .

### DISCUSSION

Recently, several papers have appeared which present information obtained by studying optical diffraction patterns of negatively stained axial unit tubules (1, 7, 8). The present report extends such studies to "free" cytoplasmic microtubules, as defined by Slautterback (17), although such

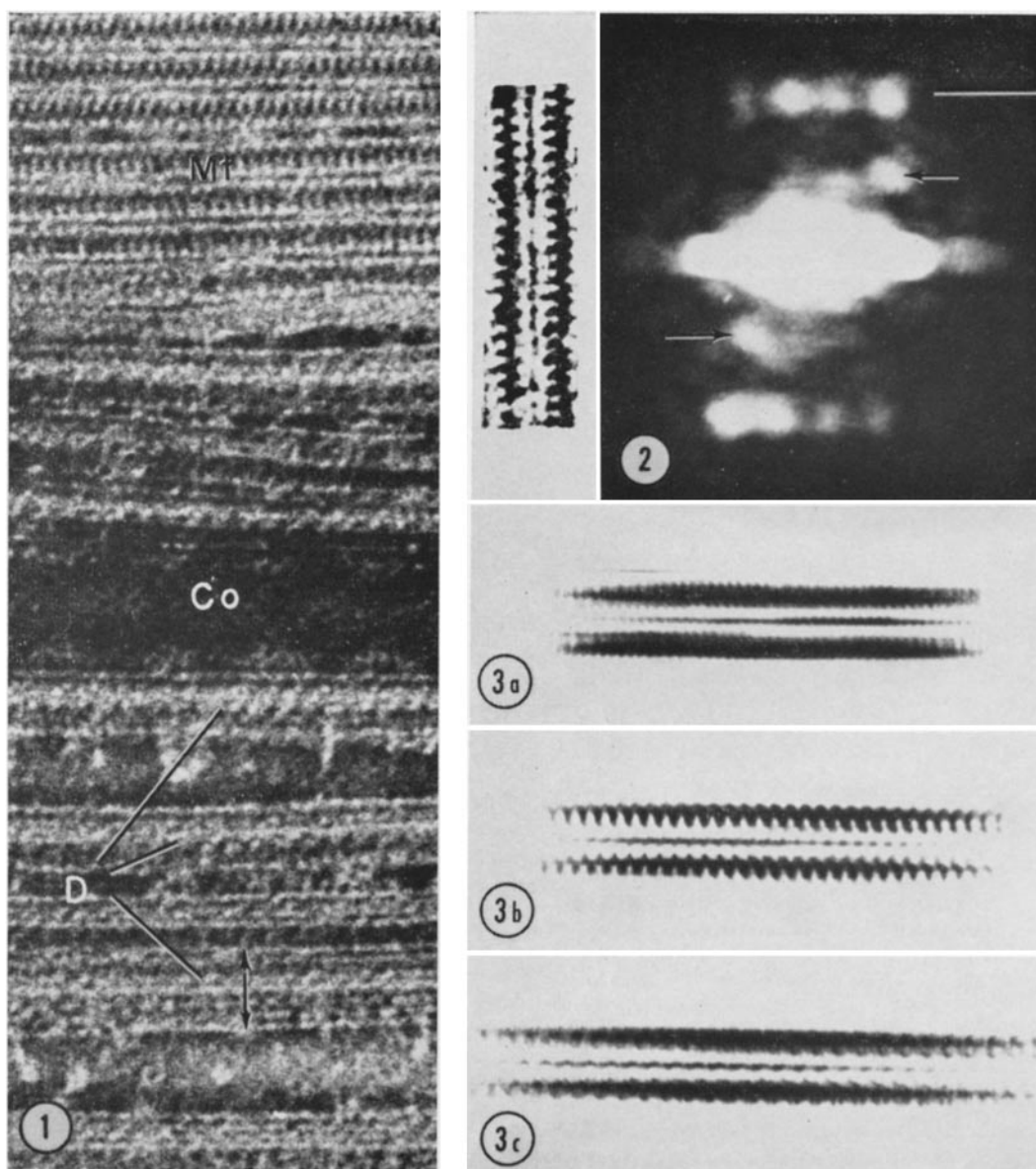


FIGURE 1 Portion of negatively stained sperm showing microtubules (*Mt*), core (*Co*) of one of two axial units, and protofibrils of flattened doublet tubules (*D*). Double-ended arrow denotes width (445 Å) of one doublet tubule. Two microtubules of those shown were masked, and positive image of mask is shown at right.  $\times 220,000$ .

FIGURE 2 Diffraction pattern obtained from mask on left. Note prominent spots on 80 Å lines and spot on 160 Å lines (arrows), which may be a harmonic effect due to diffraction of two microtubules.

FIGURE 3 *a-c* Images showing results of linear translation of above mask through 12 increments equivalent to 50 Å (*a*), 80 Å (*b*), and 110 Å (*c*). Note strong reinforcement with 80 Å repeat.

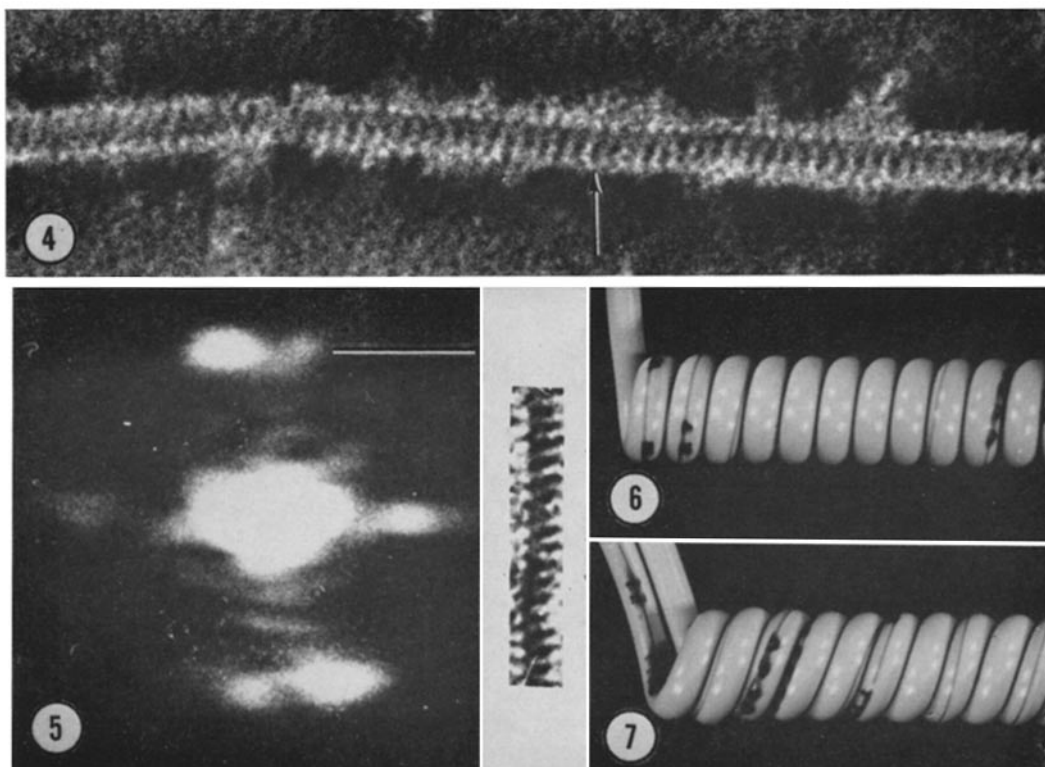


FIGURE 4 Single, negatively stained microtubule of fluke sperm which was masked in region of arrow to give mask shown below center (positive image of mask is reverse of that shown in micrograph).  $\times 290,000$ .

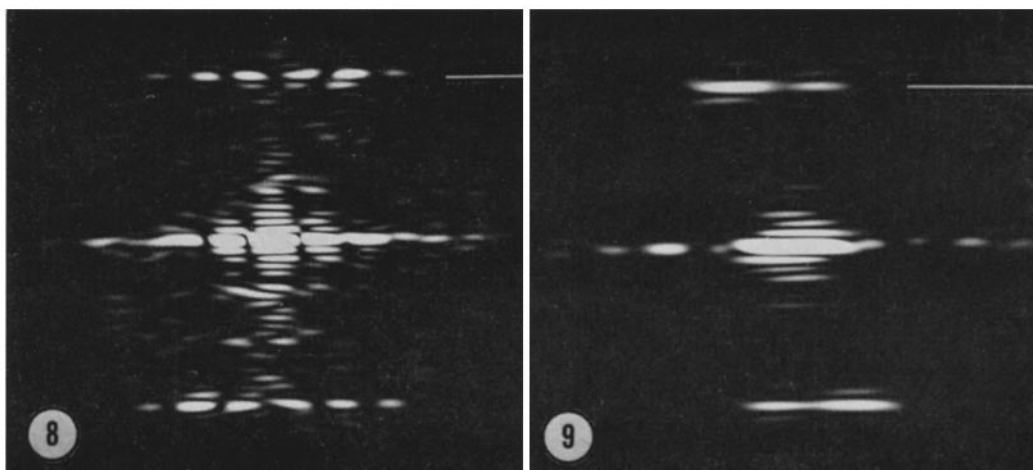
FIGURE 5 Diffraction pattern obtained from mask on right; pattern is representative of those obtained from single microtubules. Note paired spots, one more intense than other, on 80 A lines. Also, two most intense spots on 80 A lines occur on opposite sides of vertical axis, indicating helical arrangement of subunits.

FIGURES 6 and 7 Models of microtubules made from one and two strands of insulated copper grounding wire (3.8 mm in diameter) wound on a cylinder 5 mm in diameter. Inclination of helix in Fig. 6 is 7-8°, while inclination in Fig. 7 is 15-20°. See text for discussion.

microtubules in fluke sperm are atypical in several respects (3, 4).

Studies involving optical diffraction and translational reinforcement emphatically demonstrate a fundamental subunit repeat of 80 A in the microtubules examined. Further, diffraction patterns indicate that the subunits are disposed in a helical arrangement, with an inclination angle of 15-20°. The above information is in keeping with that obtained by direct examination of micrographs of negatively stained material (2, 3). The evidence thus supports the notion that the micro-

tubule is similar to a tightly coiled spring in over-all appearance (2). There is evidence that roughly spherical subunits comprise the coils of the microtubule, and the diameter of such subunits appears to be 60-65 A in transversely sectioned microtubules and about 50 A in negatively stained material (2, 3). Considering the difficulty of obtaining precise measurements in negatively stained material, it is likely that the more accurate figures are the ones obtained of sectioned material. The wall thickness of these microtubules is 60-65 A, and an examination of the literature indicates that



FIGURES 8 and 9 Diffraction patterns obtained with laser beam using masks from Figs. 1 and 4. Figs. 8 and 9 correspond to Figs. 2 and 5, respectively, in that the same masks were used. Most spots between horizontal axis and 80 Å line do not reflect periodicities in microtubules but are produced by edges of mask.

most microtubules have a wall 50–70 Å thick. With a wall thickness of 60–65 Å, and a gyre-repeat of 80 Å, it is obvious that the basic wall subunit is more ellipsoidal (60–65 × 80 Å) than spherical, with the long axis of the subunit *in situ* corresponding to that of the microtubule itself. The gyres forming the wall of the microtubule would thus be comprised of elongate subunits linked side by side to form the basic filament(s) of the helix. Longitudinal bonds between contiguous gyres would stabilize the wall in a longitudinal direction. On the basis of biochemical evidence a subunit configuration similar to the above has been suggested by Shelanski and Taylor (16) for axial unit tubules of sea urchin flagella, and it was proposed that the basic chemical subunit consists of a dimer of the 40 Å subunits seen in protofibrils of negatively stained material. Pease (14) noted transverse periodicity in negatively stained axial unit tubules of rat sperm. The interperiod spacing was about 80 Å, and Porter (15) used this and other information to propose that wall subfilaments of microtubules are constructed of units 80 Å long and 45–50 Å wide strung end to end. The latter dimension represents the approximate diameter of wall protofibrils seen in negatively stained material.

Originally, the wall of fluke sperm microtubules was visualized as being comprised of a single, tightly coiled filament (2). The construction of models indicates that it is just as likely that two

contiguous filaments, winding in the same direction, contribute to each turn of the helix. In a similar manner, Hoffman (9) has discussed the problem of filament number and gyre inclination in chloroplast microtubules of *Oedogonium*, which are obliquely striated. Figs. 5 and 7 are models constructed in exactly the same manner, except that the wall of the “microtubule” in Fig. 6 is composed of one coiled filament while that in Fig. 7 is made up of two juxtaposed filaments. In both models, the “microtubule” is scaled to a diameter of 220 Å, with the wall measuring 64 Å in width. In the models, the subunits of the wall filaments would be spherical in shape, as opposed to apparently ellipsoidal subunits in a real microtubule, although the models shown are sufficiently accurate to illustrate the point. With a single, tightly wound filament (Fig. 6), the inclination of the gyres is 7–8°, but when two filaments are wound side by side in the same direction (Fig. 7), an inclination of 15–20° is obtained. Since fluke sperm microtubules do not usually appear greatly flattened or distorted in negatively stained preparations, it is possible that the 15–20° inclination seen in diffraction patterns reflects the existence of a pair of coiled filaments forming the microtubule wall. A single filament comprised of elongate subunits 60–65 × 80 Å would produce a helix having an inclination of only about 10°, which does not fit the available data.

As in fluke sperm microtubules, diffraction pat-

terns of negatively stained axial unit tubules show a helical arrangement of wall subunits (1, 7, 8). A basic surface lattice of about  $40 \times 50$  A has been described (1, 8), reflecting the separation of the wall into longitudinal protofibrils with a center-to-center spacing of about 50 A. In more intact tubules, higher orders of periodicity were noted, one being an 80 A repeat which was assumed to be a result of perturbations ("zigzags") of the 40 A subunit repeat along the length of the protofibril. Grimstone and Klug (8) considered 480 A to be the major axial repeat in axial unit tubules of certain protozoan flagella, while Barton (1) suggests that in axial unit tubules of sperm cells of a fern the major repeat is 176 A.

Diffraction patterns of negatively stained axial unit tubules are obviously more complex than those of fluke sperm microtubules, and until more detailed patterns can be obtained of the latter, no comparisons will be attempted other than to note a helical configuration common to all patterns examined to date. As regards helices and microtubular elements, it may be worthwhile to keep in mind Crane's (5) discussion of chain formation in living systems, wherein he provides a provocative discussion on behalf of the statement: "...any structure which is straight or rodlike... is probably a structure having repetition along a screw axis."

#### SUMMARY

Both optical diffraction and translational reinforcement were used to study the periodic pattern

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of negatively stained cytoplasmic microtubules from sperm of the frog lung-fluke (*Haematoloechus medioplexus*). Information thus obtained is correlated with previous studies of these microtubules. Optical diffraction patterns show a prominent linear periodicity of 80 A, even when several microtubules are simultaneously examined and such periodicity is confirmed by translational reinforcement. Diffraction patterns show that wall subunits are in a helical arrangement, with the gyres inclined  $15-20^\circ$ . The 80 A linear periodicity and a wall thickness of 60-65 A suggest that individual wall subunits are not spherical but ellipsoidal in shape, with their long axes paralleling that of the microtubule. Consideration is given to the number of subunit chains coiled to form the wall of the microtubule, and the model best providing for a gyre inclination of  $15-20^\circ$  is one where the wall is comprised of two juxtaposed chains winding in the same direction.

The author is indebted to Ralph W. Gerchberg, Department of Electrical Engineering and Center for Research in Engineering Science, for providing diffraction patterns utilizing a laser system.

This research was supported by U. S. Public Health Service Grant AI 06448 and Career Development Award 1-K3-GM-8620-02 from the Institute of General Medical Sciences.

Received for publication 4 September 1969, and in revised form 14 October 1969.

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