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MECHANISMS OF CILIARY MOVEMENT: CONTRIBUTIONS FROM ELECTRON MICROSCOPY

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Abstract

A brief review of important contributions of electron microscopy to the study of ciliary motility is presented. The electron microscope was used to show the universality of axonemal structure of cilia, and to develop the sliding microtubule model of ciliary motility and later the switch point hypothesis to explain the conversion of sliding into bending. Unexpectedly, insights into the importance of cilia in human health have stemmed from these studies.

Key Words: Axoneme, dynein, microtubules, Kartagener's syndrome, immotile cilia.

Introduction

This article briefly reviews some of the seminal contributions of electron microscopy to the study of ciliary motility. Although they are only about 10 μm long and 0.25 μm in diameter, because of their beat, cilia have been known from light microscopy for over 300 years. At the beginning of the twentieth century some ingenious light microscopists showed that they possessed a substructure of fibrils, even though the fibrils were below the supposed limit of resolution of the microscope. One of the early triumphs of biological electron microscopy was to define the substructure more precisely and to show that the 9+2 pattern of the ciliary axoneme was universal (Manton, 1952; Fawcett and Porter, 1954). Continuity of the ciliary membrane with the cell membrane and continuity of the axonemal fibrils, or microtubules as they came to be called, with those of the basal body were demonstrated (Table 1).

Axonemal Structure is Responsible for Motility and Control of Motility

Cilia often beat out of phase with one another, producing metachronal waves. Early descriptions noted that the motion was like "a field of wheat", an incorrect analogy, because cilia operate in a fluid environment, propelling water or mucus with low Reynolds number hydrodynamics (cf. Satir and Sleigh, 1990). Nevertheless, the metachronal activity pattern could be captured by quick fixation for light microscopy (Gelei, 1926), and a second early advance was to extend such procedures to electron microscopy (Satir, 1963) (Fig. 1). It is interesting to note that the phase differences between single cilia can be captured in such images suggesting that fixation occurs in only a few milliseconds.

From the standpoint of motility, a key question in those early days of modern cell biology was: is the cilium an active organelle or is it passively moved from its base by the cell? Hydrodynamic theory said the former was true, but it remained to show that the cilium could be removed from the cell and reactivated by ATP addition. The use of ATP-reactivated axonemes pioneered

TABLE 1. Some contributions of electron microscopy to studies on ciliary structure and motility.

Date	Contribution	Reference
1952-54	The 9+2 axoneme is universal for motile somatic cilia and many sperm tails. The axoneme is surrounded by a ciliary membrane.	Manton, 1952; Fawcett & Porter, 1954
1955-57	The 9+2 pattern is related to the 9+0 pattern of centrioles, basal bodies and non-motile ciliary derivatives.	Porter, 1957
1959-63	Discovery of the dynein arms and radial spokes.	Afzelius, 1959; Gibbons, 1963
1963	Fixation of the metachronal wave at electron microscopic resolution.	Satir, 1963
1965-74	The sliding microtubule model of ciliary motility.	Satir, 1965; 1968; Summers & Gibbons, 1973; Warner & Satir, 1974
1971	Discovery of the ciliary necklace.	Gilula & Satir, 1972
1976	Human ciliary mutations cause an immotile cilia syndrome.	Afzelius, 1976
1977	Dynein is a (-) end directed microtubule motor.	Sale & Satir, 1977
1982-83	Isolated dynein is a bouquet with 2 or 3 globular heads.	Johnson & Wall, 1983
1979-89	The switch-point hypothesis: dynein arm activity during beat is non-uniform.	Wais-Steider & Satir, 1979 Tamm & Tamm, 1984; Sale, 1986; Satir & Matsuoka, 1989

by Hoffman-Berling to study motility required the complementary observation on electron microscopic preparations that the ciliary membrane was removed by detergent treatment before a complete appreciation of the role of the axoneme as the motor of the cilium was achieved. Careful analysis of sperm motility, primarily by Gibbons and colleagues, indicated that living and ATP-reactivated axonemal beat forms were virtually identical, provided that the reactivation medium was appropriate in pH, ionic strength and composition. We now know that 9+2 axoneme, the detergent-treated cytoskeleton of the cilium, not only contains the structure responsible for the generation of ciliary motility, but also the structures that respond to various second messengers in the cell cytoplasm to control the frequency and form of the beat, for behavioral responses of ciliated cells. The ciliary membrane, on the other hand, is responsible for maintaining the environment around the axoneme, particularly the Mg^{2+}-ATP concentration that permits motion to occur. The membrane also is responsible for generating the messengers, e.g., cAMP-, or changing the environment, e.g., via voltage-sensitive Ca^{2+} channels, to initiate changes in axonemal behavior. The membrane and the axoneme are in communication, sometimes directly, but mainly indirectly, through a soluble phase that is lost during detergent extraction and subsequent washing.

These conclusions have led to an intensive focus on the axoneme in terms of biochemistry, structure, mechanisms of motility and response. By two dimensional gel electrophoretic analysis, the axoneme is known to be comprised of over 200 polypeptides. The locations of only about 50 of these and the functions of even fewer are known. The known polypeptides make up the major structures of the axoneme. For example: α and β tubulin, together with an intermediate filament homolog tektin, comprise the 9+2 microtubules; the arms on the microtubules are the dyneins, each dynein consisting of heavy, intermediate and light chains (perhaps 6-9 polypeptides in all per dynein) for a total molecular weight of 1-2 x 10^6 and the radial spokes consist of at least 17 identifiable polypeptides in *Chlamydomonas*. While the understanding of the biochemistry of each structure is far from complete, the arrangement of the structures to form the intact axoneme, and in a rudimentary way, the function of each structure per se in generating motility has been delineated. It is thus possible to model the axoneme in three-dimensions by computer reconstruction techniques at a resolution approaching 4 nm (Sugrue *et al.*, 1991) (Fig. 2). Such reconstruction captures the shapes, dimensions and periodicities of the axonemal components. In *Tetrahymena*, there is a major 96 mm repeat in the axoneme, corresponding to the spoke group repeat. There are 3 spokes per spoke group. There are also four outer arms per 96 nm and a paired set of inextensible interdoublet links (Warner, 1983). More controversial are the structure of the inner arms, their

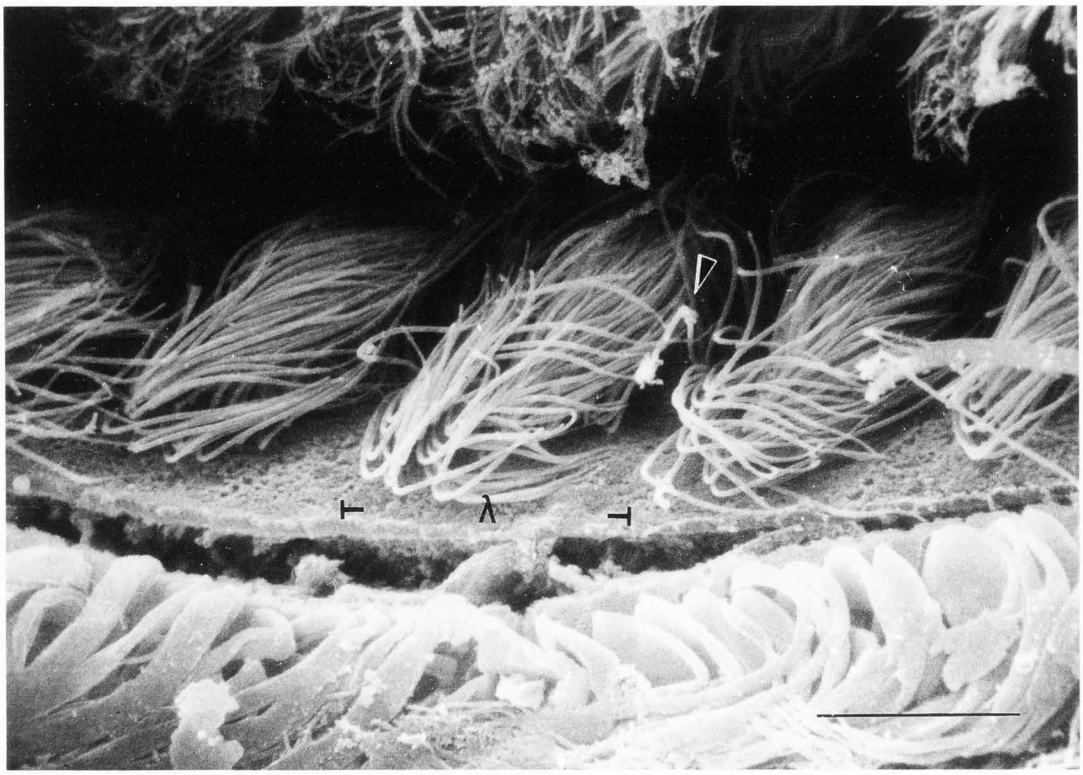

Fig. 1. Scanning electron micrograph of fixed metachronal wave of mussel gill lateral cilia. A wavelength (λ) is comprised of about 200 cilia, most of which are captured here in the recovery stroke. In one wavelength fixation has been rapid enough to capture a number of cilia at the start of the effective stroke (e.g., pointing toward the viewer at arrowhead). See Satir (1963). Micrograph from Satir and Dirksen (unpublished work). Bar = 10 μm.

possible heterogeneity and periodic repeat, as well as the placement of a possible second set of interdoublet links with a 24 nm period. Finally, the structure of the attachments to the central pair of microtubules may be species specific. In any event, the overall match between the model and actual micrographs of the axoneme is good.

The Sliding Microtubule Model: Sliding of Axonemal Microtubules by Dynein Arms is Transduced into Ciliary Bending

Fixation of the metachronal wave made it possible to examine what was essentially an identical cilium stopped in different beat positions. With fixation, structural changes in the axoneme with time were displayed as changes with distance along the wave. In this way, it could be shown that the axonemal microtubules changed position as beat proceeded in a manner completely consistent with the generation of bends by a sliding microtubule mechanism (Satir, 1968; Warner and Satir, 1974). The amount of bending was related to the sliding displacement of any microtubule with respect to a fixed axis by a constant of proportionality dependent on the distance between the microtubule and the axis, essentially determined by axonemal diameter. This result has been confirmed on moving ATP-reactivated axonemes (Brokaw, 1989, 1991).

Sliding of microtubules was directly and dramatically shown in the light microscope by Summers and Gibbons (1971, 1973) using trypsin-treated axonemes. Sale and Satir (1977) extended these results with electron microscopy to show that all active sliding has a single polarity. In producing sliding, dynein arms along any one doublet N always push the next doublet (N+1) tipward; by Newton's third law, N moves baseward. This demonstration that axonemal dynein is universally a (-) end directed microtubule motor has stood the test of time (Fox and Sale, 1987), and it also applies generally to cytoplasmic dynein. Scanning transmission electron microscopy images of isolated dynein showed that the molecule responsible for sliding was a 2 or 3 headed bouquet (Johnson and Wall, 1983), but one head alone is sufficient for sliding *in vitro* translocation arrays and one head alone probably interacts with the adjacent microtubule in the axoneme.

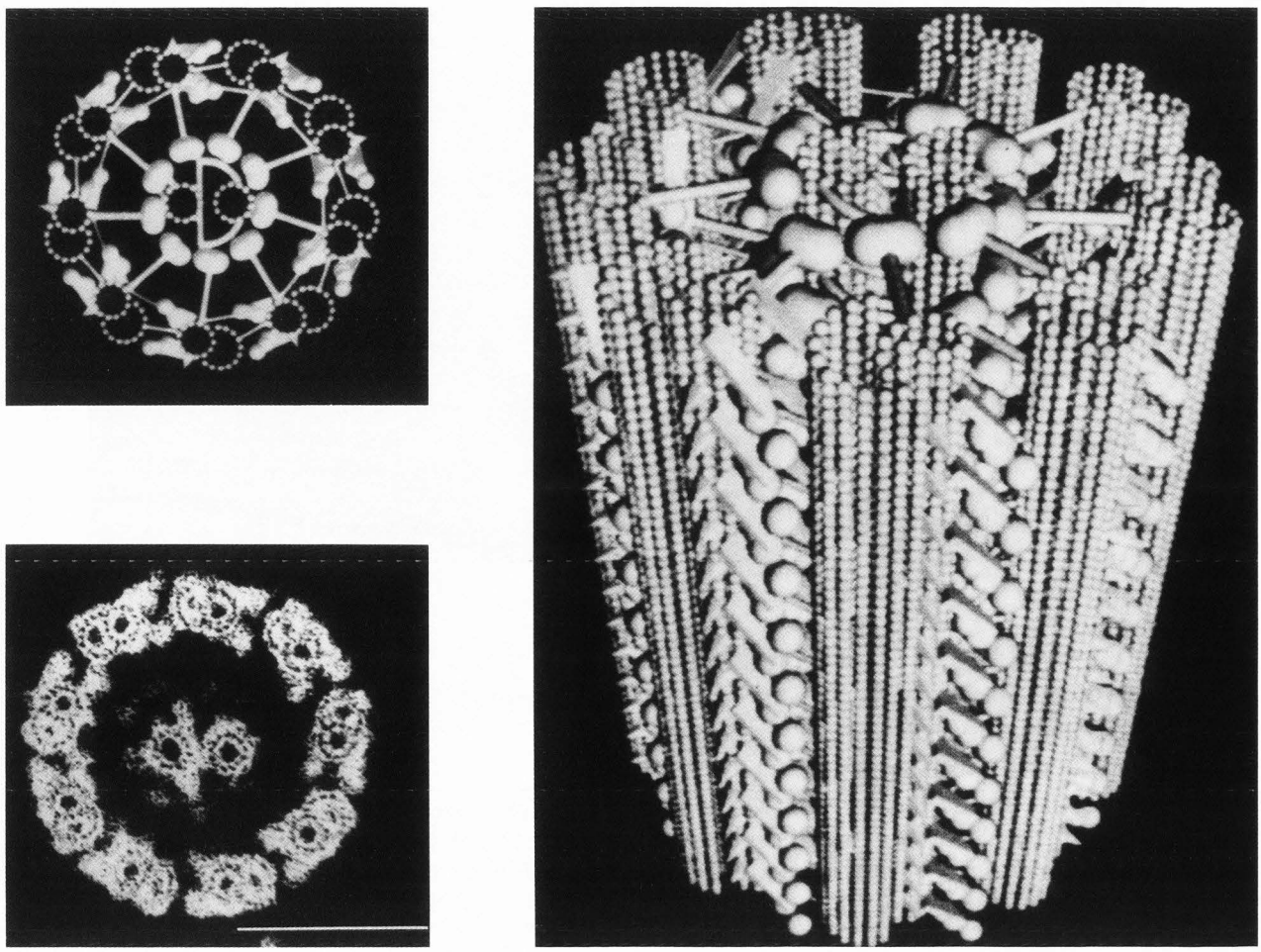

Fig. 2. Computer reconstruction of the ciliary axoneme. **Left: Above**. View of model base to tip. **Below**: *Tetrahymena* axonemal cross-section printed at same magnification. Bar = 0.1 μm. The computer model captures the forms and dimensions of the major axonemal components at a resolution approaching 4 nm. **Right**: A three-dimensional reconstruction of a portion of the axoneme from Sugrue *et al.* (1991).

After trypsin treatment, when sliding is induced by ATP, the axoneme does not beat. Instead, the microtubules in an individual axoneme slide for nearly their entire length, until they overlap one another only by a short distance of a few tenths of a micrometer. Sliding occurs at rates of 1-20 μm/sec as a one time event. In contrast to the irreversible 10 μm or so of sliding seen after trypsin treatment, sliding in an intact axoneme is recurrent at frequencies of beat, normally between 10-50 Hz, i.e., every 20-100 milliseconds. Sliding is strictly coupled to bend formation and is very limited, usually to about 0.1 μm. However, the rate of sliding, say 0.1 μm/10 msec, is about the same as after trypsin treatment. For sliding to recur, any doublet that moves in one direction must be brought back to its starting position over and over again. Thus, active sliding on a doublet must be balanced by a passive sliding, where the dynein arms are not active, and the doublet moves the other way.

The sliding microtubule model suggests that in the intact axoneme, as sliding proceeds, it is resisted by a series of linkages, including: (1) attachment between axonemal spoke heads and central pair projections and (2) circumferential interdoublet links, that limit its extent, and convert the sliding to local bending. Trypsin-treatment destroys or significantly weakens these linkages (Summers and Gibbons, 1973) permitting each doublet with active arms to slide in an unrestricted manner, as far as is possible.

Structural Deficits in Ciliary Mutants as Defined by Electron Microscopy can be Understood in Terms of the Sliding Microtubule Model

Mutant axonemes lacking dynein arms, but also spokes or central microtubules, have been defined by

electron microscopy. The sliding microtubule hypothesis predicts that all of these will be paralyzed. Although mutants lacking spokes or central microtubules may still slide, sometimes without trypsin treatment, they will not be able to convert this sliding into systematic bend generation.

Paralyzed cilia are reasonably obvious mutations in a single celled organism such as *Chlamydomonas*. Not as obvious is the understanding that a series of human mutants characterized by chronic bronchitis, rhinitis, otitis media and male infertility (related symptoms, first called Kartagener's syndrome) are often due to defects of the ciliary axoneme (Afzelius, 1979). In man, cilia line parts of the respiratory and reproductive tracts. They are also found in ependymal cells lining the ventricles of the brain and the human sperm tail is essentially a single long cilium with accessory structures. When it was realized that the symptoms of Kartagener's syndrome were due to paralyzed cilia with the same general structural defects as are present in *Chlamydomonas* mutants, the terms 'primary ciliary dyskinesia' or 'immotile cilia syndrome' were used to describe this pathology (Afzelius, 1976, 1979; Sturgess *et al.*, 1979, 1980; Baccetti *et al.*, 1981; Nielsen *et al.*, 1983). The sliding microtubule model, therefore, provides insight into these human pathologies.

The Switch Point Hypothesis: Beat is Produced by a Changing Pattern of Dynein Arm Activity in the Axoneme

A simple formulation, the switch point hypothesis, accounts for the coupling of ciliary beat to successive active versus passive sliding events necessary for recurrent motion. The hypothesis, which is based on the polarity of sliding at the axonemal tip, assumes that about half of the axoneme is active at one time to produce the effective stroke; then dynein arms on this half of the axoneme becomes inactive and non-cycling, while those on the opposite half become active to produce the recovery stroke. The halves are set up so that the axoneme generates two oppositely oriented bends and thus beats back and forth. The hypothesis makes several predictions that are testable (Satir and Sleigh, 1990). Several tests involve electron microscopy. A major prediction is that two types of arms will be found in terms of activity: cycling, active and non-cycling, inactive. Inactive arms will be functionally detached from doublet N+1 so that N and N+1 can move passively with minimal resistance; active arms will have some phases of attachment and some of low affinity or functional detachment, perhaps in a repetitive fashion. The two states can be assayed in negative stain preparations, after treatment of isolated axonemes with a non-hydrolyzable ATP analog

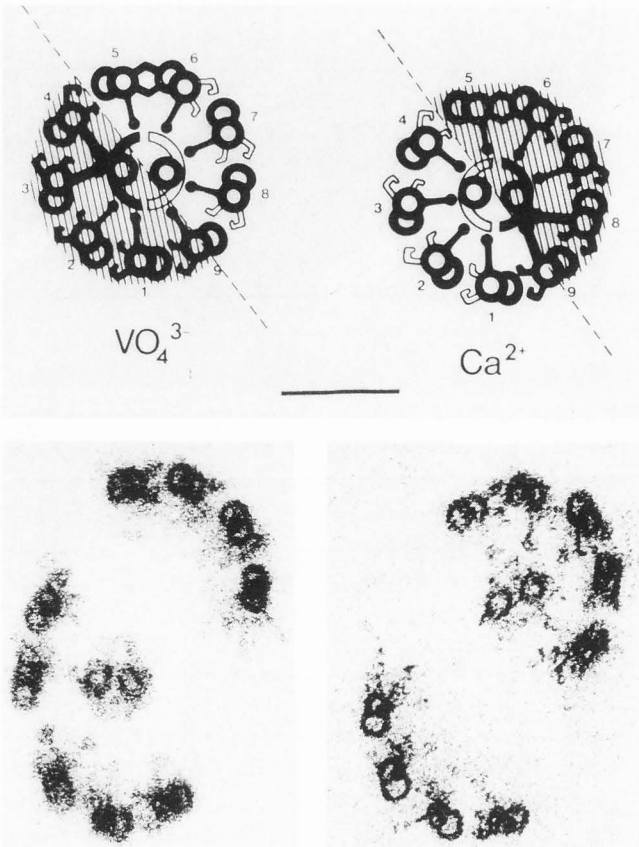

Fig. 3. Axonemal splitting in 'Hands Down' versus 'Hands Up' mussel gill lateral ciliary axonemes suggests that different halves of the axoneme have active arms. **Above**: Predictions of activity in axonemes arrested by the ions shown. **Left**: 'Hands Down'; **Right**: 'Hands Up'. Shaded areas and black arms indicate active doublets, predicted by the switch point hypothesis. **Below**: Split axonemes: **Left**: 'Hands Down'; **Right**: 'Hands Up'. The central pair splits with the active half axoneme (from Satir and Matsuoka, 1989). Bar = 0.1 μm.

(Spungin *et al.*, 1987). Further, in some axonemes, switching between active half axonemes can be paralyzed by certain inorganic ions, so that the beat stops because the axoneme arrests in one position (Wais-Steider and Satir, 1979; Reed and Satir, 1986; Satir and Matsuoka, 1989). In this position in every arrested axoneme, the same defined set of doublets should have active arms, while a second defined set has inactive arms. Now, if these axonemes are trypsin-treated in the presence of inhibitor so that switching cannot occur, the doublets with inactive arms should be unable to slide when ATP is added. The doublets with active arms will slide away, splitting the axoneme into sliding versus

non-sliding halves. This experiment has been performed in several different systems (Tamm and Tamm, 1984; Sale, 1986), most completely by Satir and Matsuoka (1989). Splitting of the paralyzed axonemes is consistent with the switch point hypothesis (Fig. 3). One important consequence of the hypothesis is that changes in ciliary behavior only require changes in the timing of the activity pattern of the two half axonemes. Satir and Sleigh (1990) indicate how this might apply to single-celled organisms or to mammalian ciliary epithelia.

Conclusions

Electron microscopy has been intimately involved in the dissection of the mechanics of ciliary motility, first by defining the axoneme and its components, then by suggesting and supporting the sliding microtubule model of motility. Finally, the switch point hypothesis of beat generation depends in large measure on proof involving fine structure analysis of the axoneme. Unexpectedly, insights into the importance of cilia in human health have stemmed from these studies. A more complete understanding of the motile mechanism might lead to methods of intervention that would increase the efficiency of cilia in respiratory clearance in situations where this is impaired. It is likely that electron microscopy will continue to play an important role in providing such understanding.

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Discussion with Reviewers

B. Afzelius: You state that mutants lacking central tubules will not be able to convert sliding into systematic bend generation. This is certainly true of most mutants lacking the central tubules but may not be generally true. Normally motile (or nearly so) mutants will not be found by investigators searching for mutants, but spermatozoa from several animal species move very rapidly without inner tubules, e.g., the myzostomid worm (Afzelius BA: J. Ultrastr. Res. **83**, 58, 1983), the eel (Gibbons B, Baccetti B, Gibbons IR: Cell Motility **5**, 333, 1985) or the Asian horseshoe crab (Ishijima S, Sekiguchi K, Hiramoto Y: Cell Motil. Cytoskel. **9**, 264, 1988). Any comments?

Author: My emphasis in this paper is about mutants with regard to sliding-bending conversion. The sperm tails mentioned by Prof. Afzelius are not mutants of a wild type 9+2 axoneme, except possibly in an evolutionary sense, by natural selection.

B. Afzelius: Your Figure 1 shows lateral cilia from the mussel gill. It appears to me that the number of cilia along a wavelength and thus in a metachronal wave is much less than 200. Most of the cilia shown in the five assemblies are in synchrony rather than in metachrony. Please comment.

Author: Regarding Fig. 1, TEM of comparable images and calculation shows that there are about 200 cilia in a wavelength of about 10 μm. Also, analysis shows subtle differences between adjacent cilia suggestive of movement. Why the SEM impression is different I am not sure, but the Figure Legend is accurate.

H. Machemer: Another important step was the discovery that the central core of a cilium can rotate whereas the periphery slides unrotated [Omoto CK, Kung C (1980) Rotation and twist of the central-pair microtubules in the cilia of *Paramecium*. J. Cell Biol. **87**:37-46; Omoto CK, Witman GB (1981) Functionally significant central-pair rotation in a primitive eukaryotic flagellum. Nature (London) **290**, 708-710]. The morphological basis of central-core rotation, only one central microtubule connects to the basal granule, had been established previously [Dute R, Kung C (1978) Ultrastructure of the proximal region of somatic cilia in *Paramecium tetraurelia*. J. Cell Biol. **78**, 451-464].

I would have expected in this review to see the "switch-point hypothesis" (1979) discussed in the light of contemporary models [Machemer H (1977) Motor activity and bioelectric control of cilia. Fortschr. Zool. **24**, 195-210; Baba SA, Mogami Y, Nonaka K (1990) Discrete nature of flagellar bending detected by digital image analysis. In *Biological Motion*, Alt W, Hoffmann G (eds.). Lect. Note Biomath. 89: 145-154] and their evidences. The rotary-sliding engine model by Machemer (1977) addresses all major implications of unidirectional active sliding within a cilium.

Author: Rotation of the central pair may be true for protozoa but it is definitely not true for ctenophores (Tamm and Tamm) or mussel gill lateral cilia and probably not true for other metazoa.

Prof. Machemer's model is a variation of the "switch-point" hypothesis for which experimental tests have not been proposed.