Self-reference, closed circuits and the phototactic sensorimotor mechanism of the unicellular alga *Euglena gracilis*. An application to the study of nonlinear dynamics in microtubular structures.

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Abstract.

Contemporary biology still lacks a model capable of accounting for even the most basic phenomena involving microtubules (MT) such as for example intracellular transport and ciliary beating, not to speak of the function of MT in in sensory and nerve cells.

As an example, vertebrate photoreceptors contain a cilium whose dynamic function is still unknown.

A new model of microtubular dynamics, developed by the author together with P. Zaborski and J. Tuszynski (Insinna et al., 1996) and based on classical nonlinear physics, is capable of accounting for most of the phenomena associated with cell motility.

Additionally, its heuristic capabilities contribute to shed a new light not only on the phototactic behavior of the Protozoan *Euglena gracilis* but also on the dynamic role of MT in vertebrate photoreceptors and their specific responses.

Some unicellular organisms such as *Euglena gracilis* display in fact simple perceptive functions (phototaxis) which imply the use of a primitive photoreceptor.

This unicellular offers the unique possibility to study the function of MT in simple forms of vision, laying ahead of the complexity of more sophisticated photoreceptor systems.

Euglena gracilis appears to be the prototype of a self-referential, dynamical sensory system, whose study might allow us to understand the evolution of more complex perceptive systems.

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1 Nonlinear Dynamics and MT

The presence of microtubules (MT) in the cytoskeleton of eukaryotic cells is associated with dynamic phenomena, ranging from the beating of cilia and flagella to chromosome movement during mitosis (Dustin, 1984, Hyams and Lloyd, 1993).

Synchronization of dynamic cell processes involving MT and MAPs (microtubule associated proteins) such as dyneins and kinesins has not yet been entirely elucidated. (for review see Gibbons, 1988; Vale, 1987 and 1993).

Following a previous model of Tuzsynski et al. (1995) and in order to account for contraction/motility processes in eukaryotic cells, it has recently been suggested that the sequential activation of motor proteins (ATPases) is the result of charge transfer occurring inside the microtubular lattice. The mechanism is based on the occurrence of electron transfer via soliton-like kinks inside the MT lattice (Insinna et al. 1996).

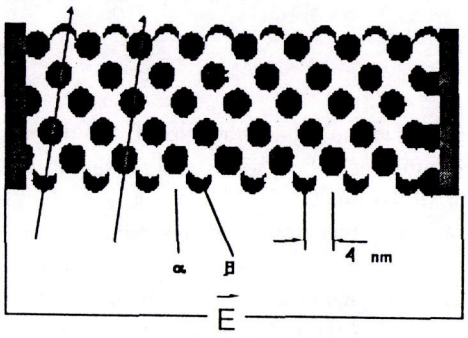

Fig. 1. MT structure. The α and β globular tubulins are arranged in the form of a hollow cylinder about 240 Å in diameter. Charge transfer is suggested to occur in the lattice either along an helical or linear pathway inside an electric field. Modified from Insinna et al., 1996.

In stable MT, current flow is suggested to occur along MT protofilaments or along specific helical paths of the microtubular lattice, provided the MT fiber is placed within an electrically closed circuit with an electron source and electron sink at its extremities (fig. 1).
The existence of this basic condition for the proper functioning of stable MT structures is perhaps best exemplified in vertebrate photoreceptors. There, a cilium establishes a connection between the inner and the outer segment (fig. 2). the role of the ciliary structure has not yet been elucidated. In the present model, it works as a microtubular motor (MTM) responsible for protein transport (disk renewal) and for signaling to the synaptic terminal.

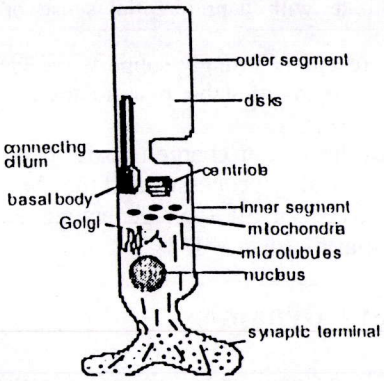

Fig. 2. Schematic representation of a vertebrate rod photoreceptor. The outer segment containing the disks carrying the rhodopsin molecules is connected to the inner segment via an immobile cilium lacking the central MT pair.

Strongly supporting the model exposed here, a "dark current" (ionic current) has been observed to flow in vertebrate photoreceptors from the inner towards the outer segment in the absence of light. The "closed circuit" configuration allows for the maintenance of a correct polarization of the MT structure and thus for proper functioning of the cilium as a transport and signaling device.

The cilium thus works as a polarized charge transfer (semiconductor) device and its equivalent electrical circuit can be drawn as in fig. 3.

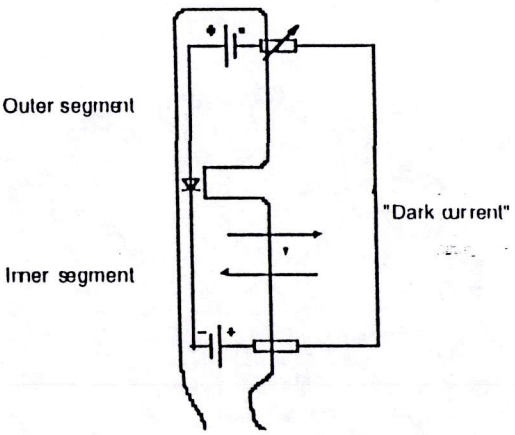

Fig. 2. Electrically equivalent circuit of a vertebrate photoreceptor. The cilium is

considered to work as a diode with a preferential sense of conduction. The "dark current"
observed in vertebrate photoreceptors strongly supports the idea of the necessity of an electrical field for proper functioning of stable microtubules.

It has tentatively been postulated that charge transfer-dependent protein activation is at the base of all MT-driven motility processes and that MT are the cloking devices capable of coordinating the activity of all force-generating enzymes. MT thus truly deserve the name of microtubular motors (MTM).

2 Flagellar and Ciliary Dynamics

Cilia and flagella are dynamic organelles visible in many eukaryotic cells. Many unicellulars, for example, possess on their surface flagella as locomotory organs that beat in a regular fashion with frequencies up to tens of Hz (Sleigh, 1974).

The charge transfer model is heuristic enough to account also for ciliary and flagellar beating. It suggests that the maintain of continuous charge transfer inside the axoneme is achieved by a potential difference (bias) existing between the tip of the axoneme and the cell's interior. In most cilia and flagella, one can in fact observe special membrane domains separating the ciliary membrane from the negative potential in the cell's interior and sophisticated capping structures which terminate the distal ends of the axonemal MT (for review see Dentler, 1990). The model further implies that charge transfer inside the MT subfibers and the central MT pair is responsible for the sequential activation of the motility proteins (the dynein arms). Fig. 4. Finally, it advances that, in addition to the activation of the motor domain (the head), a conformational change occurs at the distal part of most motor proteins. This conformational change distally of the motor domain has been widely implemented by the cell to solve all synchronization problems inherent in ciliary and flagellar dynamics.

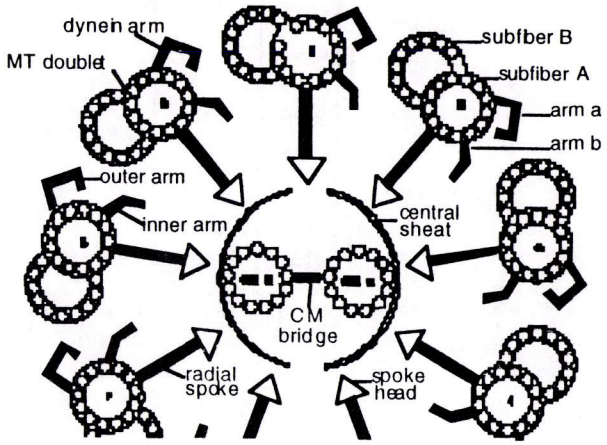

Fig. 4. Schematic representation of the axonemal components of a cilium . The cross-section is viewed from base to tip. CM, central microtubule. Modified from Warner and Satir, 1974 and Insinna et al., 1996.

This conformational-change mechanism plays a major role in the radial spokes connecting the central pair MT to the peripheral doublets (fig. 4). The radial spoke-head ATPase is needed for signaling to and control of the MT doublet to which the radial spoke is attached distally. The conformational change occurring in the distal part of the radial spokes works as a gating mechanism as in semiconductors devices. When inactivated, the radial spokes block the current flow inside the corresponding MTB subfibers as long as the radial spoke head is not disconnected from the radial sheath of the central MT pair (CM3 and CM8) through ATP hydrolysis.

The gate mechanism also involves the participation of the radial or inner sheath, consisting of two rows of projections on each central MT (Witman et al., 1976; Warner, 1976). The role of these components is to distribute charge transfer signaling from the central pair simultaneously to several radial spokes of the axoneme. A schematic representation of the gating mechanism is shown in fig. 5.

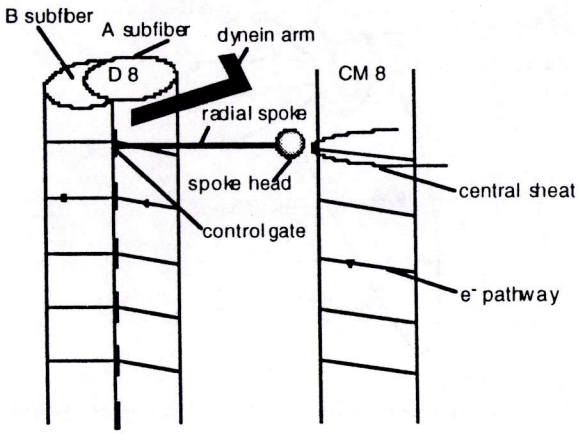

Fig. 5. Gating mechanism in cilia. Charge transfer inside the central MT (CM8) and conformational changes in the central sheat and radial spokes (via ATP hydrolysis) are responsible for synchronization of dynein arms activity. Modified from Insinna et al., 1996.

This is not the place to describe in detail the entire dynamics of the flagellum. Suffices to say that the dynein arms and the radial spokes are helically disposed along the axonemal structure. Combined with the sequential charge transfer activation of the motor proteins, this helical disposition results in a progressive spreading apart of a winding of the axonemal components under the action of CM 3 and MT1 to 5. .Because of the mechanical constraints inherent in the axoneme (nexin bridges between the doublets) a bending will occur in this particular zone of the flagellum. The progressive spreading apart of the imaginary winding will continue along the entire structure through CM8 and MT6 to 9. A helical bending wave thus propagates progressively along the flagellum. According to this model, only a slight local sliding between MT is needed to produce the bending wave.

3 *Euglena gracilis*

The protozoan alga *Euglena gracilis* offers the unique possibility to study the function of MT in a simple form of behavioral response (phototaxis), in connection with a primitive sensorimotor mechanism. Its study can give us clues about the function and evolution of the more sophisticated neural systems of vertebrates.

In *Euglena*, the locomotory flagellum (Emergent Flagellum, EF) is of the 9+2 type and is made up of nine doublet MT and of a central MT pair, as the majority of motile MT structures (fig. 4). A membrane invagination (the reservoir) contains the proximal part of the flagellum and its distal part emerges from it through a so-called canal. Another flagellum (Non-Emergent Flagellum - NEF), in most cases immobile and much shorter, originates from a second basal body and remains confined inside the reservoir (Moestrup, 1982). Fig. 6. A pulsating vacuole is located at the base of the flagella, close to the reservoir.

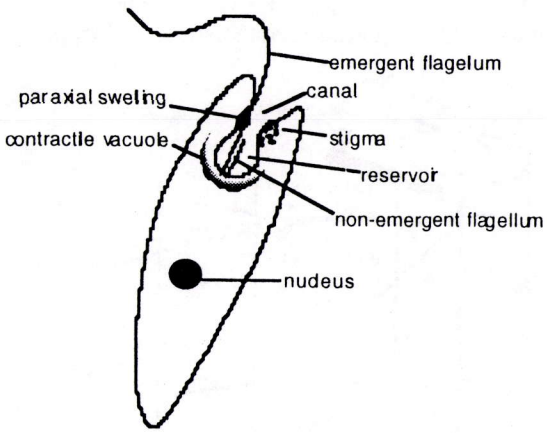

Fig. 6. *Euglena gracilis'* major organelles. Modified from Insinna et al., 1996.

In the motile flagellum, an hollow rod-like structure with a diameter of 90 nm, the paraxial rod (PAR), has been observed adjoining the axoneme, along its entire length. The PAR is composed of seven 22-nm filaments coiled into a 7-start left-handed helix (Hyams, 1982; Moestrup, 1982). Some MT doublets of the flagellar axoneme (probably MT 1, 2 and 3) are linked to goblet-like projections emanating from the PAR (Bouck et al., 1990). Fig. 7. ATP-ase activity has also been detected in the rod of *Euglena* (Piccinni et al., 1975).

A visible ovoidal protuberance, the paraflagellar swelling (PFS) is located on the emergent flagellum somewhere between the reservoir and the canal. At the level of the PFS, the paraxial rod seems to uncoil and completely surround the swelling (Gualtieri et al., 1990). In *Euglena*, the PFS contains as many as 1.5×10^7 rhodopsin molecules forming a crystalline-like lamellar structure (Gualtieri et al. (1992; Leedale, 1967; Wolken, 1977; Piccinni and Mammi, 1978). Thus the PFS is the active location for photoreception which was long searched for (fig. 6).

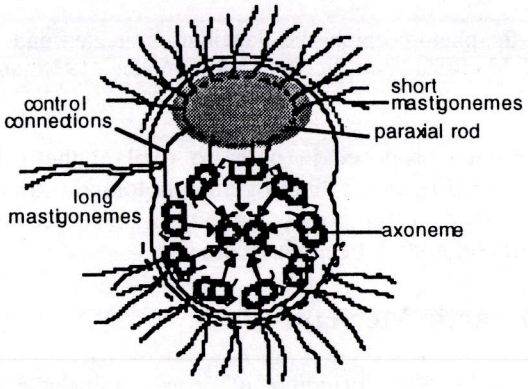

Fig. 7. Cross-section of *Euglena gracilis'* emergent flagellum. Control connections link the paraxial rod (PAR) to some of the A-subfibers of the axoneme. Modified from Buck et al. 1990 and Insinna et al., 1996.

The stigma, an orange-red organelle made of spheroidal granules located at the canal level and optically in line with the PFS has, instead, only the role of a shading device (Feinleib and Curry, 1971; Feinleib, 1985; Gualtieri et al., 1989).

The protist moves by the propulsive force of the 50 μm long beating flagellum EF in which roughly helical bending waves propagate along its structure. The 50 μm long and 10μm wide cell moves in a helical path by spinning along its axis with a frequency of 1.2 to 1.8 Hz. In the phototactic strategy, the organism changes its direction in response to a light stimulus and swims either toward (topotaxis) or away (negative phototaxis) from the light source. Positive phototaxis occurs through a series of corrective responses generated by the cell every time the stigma casts a shadow on the photoreceptor (Jennings, 1906; Mast, 1911 and 1914). The cell usually swims leaving its photoreceptor permanently illuminated (Fig. 8). When the light beams are shaded by the stigma, the cell responds with an erection of the flagellum (bending reaction) which results in a change of direction. Bancroft (1913) noted that the stiffening of the flagellum is proportional to the shading time.

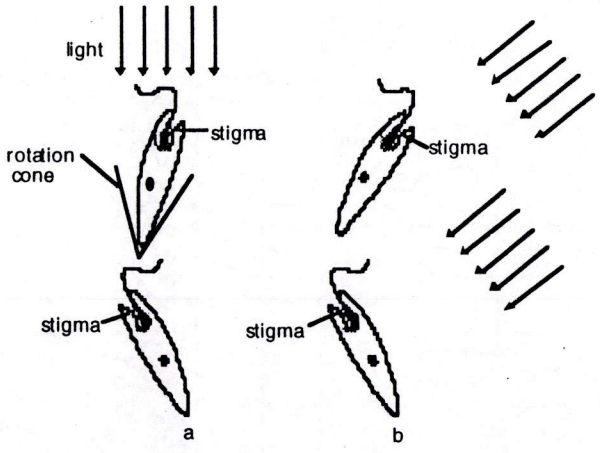

Fig. 8. *Euglena gracilis'* phototactic behavior according to Mast (1917). The cell swims toward the light source as long as the incoming light is within the rotation cone. When the

stigma casts a shadow on the photoreceptor, the flagellum is erected and the cell changes its direction accordingly. Modified from Piccinni and Omodeo, 1975 and Insinna et al., 1996.

Several models have been proposed in order to explain the role of the above organelles in connection with *Euglena's* "intelligent" phototactic strategies (for review see Insinna et al., 1996). However, the previous attempts failed because the molecular dynamics of the flagellum had not yet been elucidated.

4 *Euglena's* Phototactic Mechanism

It has been assumed that the basic principle of charge transfer control previously postulated for the radial spokes of cilia and flagella has been implemented in *Euglena's* photoreceptor architecture in a similar way. This means that the projections connected to the doublets 1 to 3 of the axoneme (fig. 7) may be considered to work as gates for the control of the axonemal current. It has further been assumed that activation of the *gate projections* happens through distally directed charge transfer occurring inside the helically disposed PAR filaments.

This leads to a coherent model of the unicellular's response to light stimuli. Thus *Euglena's* phototactic capabilities may now be outlined as follows (fig 8 and 9) :

The incoming light bleaches the rhodopsin molecules contained in the paraflagellar swelling. An enzymatic cascade, following the isomerisation of 11-cis retinal group to all-trans-retinal, induces changes in the charge transfer capability of PAR filaments.

As previously mentioned, ATP-ase activity has been detected in the paraxial rod of *Euglena* by Piccinni et al. (1975). Charge transfer activates the ATPase of the gate projection on the paraxial rod. ATP hydrolysis induces a conformational change in the distal part of the gate projection (connected to the axonemal MT doublets) thus opening the conduction path. The current may now flow inside the axonemal doublets. This may occur as long as electron flow in the PAR continuously activates ATP hydrolysis. Both the charge transfer inside the PAR filaments and the subsequent ATP hydrolysis in the rod projections are light-dependent.

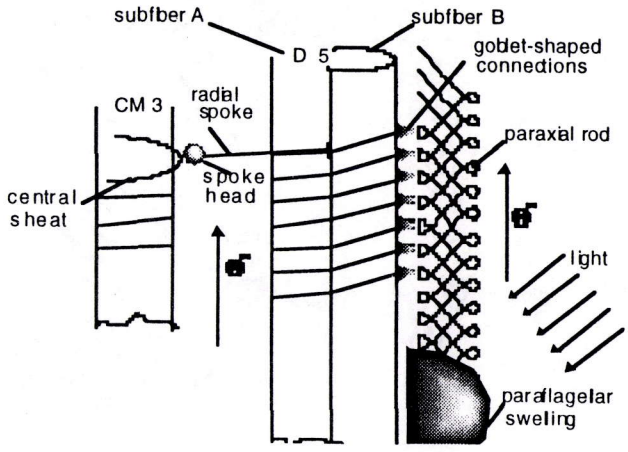

Fig. 9. Phototactic response control mechanism in *Euglena gracilis*. The paraxial rod is shown with only three fibers instead of seven. Modified from Insinna et al., 1996.

In order to achieve more efficient response, the control pathway probably involves more than one MT doublet as shown in fig. 7. Every time light impinges on the detector, the current flow in the axonemal MT doublets is proportionally increased and so is the beat frequency of the flagellum. Casting a shadow on the stigma produces instead a decrease in ATP hydrolysis and current flow in the MT doublets with a subsequent progressive stiffening of the corresponding axonemal components. The beating pattern of the flagellum and thus the cell's swimming path are modified accordingly.

The stiffened fibers directly act on the beat form of the flagellum so that it can work like a rudder. Therefore, shadowing of the PFS by the stigma helps Euglena find the direction of the light beam. The right trajectory is kept by means of successive corrections involving the flagellum beat direction.

Euglena's phototactic response to a sharp increase in light intensity consists in a shock reaction (phobotaxis) with a subsequent negative phototaxis. The flagellum is first completely straightened out and the cell subsequently moves backward during a short period (Bancroft, 1913). The present model can also easily explain how this occurs.

Upon intense illumination, the sudden bleaching of the rhodopsin pigments completely stops charge transfer in the PAR. Consequently, all the gates connected to the relative MT doublets are closed, i.e. all electronic circuits along the entire length of the concerned MT axonemal doublets are switched off. This results in complete stiffening of the flagellum. Subsequently, the Protozoan swims away from the light source via a depolarization of the cell membrane which induces a reversal of the MTM (for more details see Insinna et al., 1996).

5 Microtubules, Evolution and Autonomy (Self-Reference)

Eakin (1968) advanced that vertebrate photoreceptors and, by extension, all sophisticated sensory neurons implementing ciliary structures, are the result of an evolutionary pathway that has its origins in *Euglena*. Therefore, this Protozoan has been chosen on purpose for it is an ideal model to understand how MT have been utilized by evolution to generate fast adaptive responses to environmental stimuli in primitive forms of life.

We have seen here how, through a pragmatic application of the basic MTM model, it has been possible to give a plausible account for the structure and function of *Euglena's* perceptive mechanism. To sum up, we can draw, at this point, following conclusions :

- First, classical nonlinear dynamics and closed electrical circuits are fully qualified to produce a consistent and heuristic model accounting for MT functioning and related protein activation and synchronization (although quantum phenomena such as electron tunnelling should not be excluded and are most probably involved).

- Second, *Euglena* confronts us with a primitive form of a sensory neuron and related autonomous sensory-motor reaction. Its analysis leads to the following tentative description of an evolutionary pathway culminating with the perceptive capabilities of vertebrates : Microtubules é centrioles é ciliated cells é sensory neurons é neuronal networks é brain. *Euglena's* instinctive behavior resulting from its automated, self-referential sensory-motor capabilities, i.e. its responsiveness, might be assimilated to a primitive form of awareness and self-referential behavior. darkness.

- Third, and last, it may be advanced here that the bundles of MT inside the axons and apical dendrites of nerve cells form "intraneural networks" which determine the global firing characteristics and the specific dynamics of neurons (fig. 10).

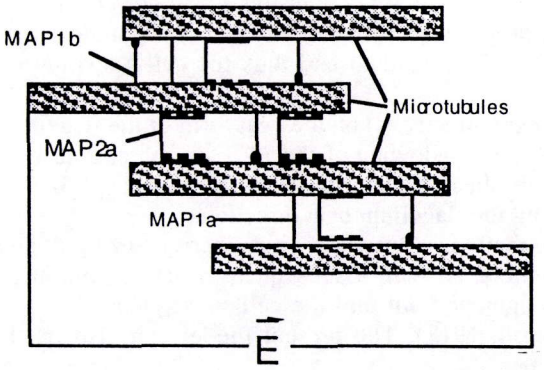

Fig. 10. Theoretical representation of an intraneural network as inferred from the present model. MAPs are assumed to control the charge transfer capabilities in MT.

Microtubule associated proteins MAP1a MAP1b, MAP2a and MAP2b could, in fact, play the same role of charge transfer control proteins similar to the connections we have seen to exist in Euglena between the PAR and the flagellum or the radial spokes in cilia (Müller et al., 1993, Matus, 1993). Both MAPs are intensely phosphorylated during brain activity (MAP1a in dendrites and MAP 2a and 2b in axons). This energy-consuming biochemical activity is probably linked to a charge transfer control mechanism responsible for the global characteristics of the MT intraneural network.

The intermicrotubular connections can be considered as necessary elements of an inherent biocomputing function of MT, as already suggested by Hameroff and Hameroff and Watt (1982, 1987) achieved via massively parallel connected MT implying the possibility of regulatory feedback loops within the axon (and dendrites). The present model should deserve a closer look from the experimental viewpoint.

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