Investigating the click-stop mechanism in the diatom *Corethron criophilum* :

optical and electron microscopy as well as micro-manipulation

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1 Diatoms

Diatoms are unicellular, eukaryotic microorganisms which are perhaps the most widespread group of phytoplancton on the earth. They occur in all aquatic ecosystems and even in some terrestrial environments (on moist surfaces of rocks and some plants).

There are between 100000 and a million species. A good estimate may never be known because there are many cryptic species out there that are only recognisable through their genetic sequences or by careful life history/mating studies.

The smallest are about 2-3m and the largest 0.1 - 0.2 mm (or even more) and visible with the naked eye. They are photosynthetic organisms although few can live heterotropically in the dark when they are supplied with a suitable source of organic carbon.

Diatoms play an essential role in the geochemical cycling of carbon and silica. They are used for studies on the climate and on environmental change.[1]

1.1 The diatom cell

The diatom cell contains all the organelles which occur in other eukaryotic algae but they have a silicated cell wall which is unique. The impregnation with silica ($SiO_2 \cdot H_2O$) is the reason for their succes in evolution. The earliest known fossils of diatoms date from the early Jurassic (about 185 million years ago) but an earlier origin is suggested (maybe after the mass extinction at the end of Perm).

The cell wall consists of two units called valves which lie at each end of the cell. The valves are linked together via the girdle region. To enable substance exchange with their environment there are pores or slits in the cell wall because the valves fit together very closely.

The wall also contains organic material that forms a thin coat around the valves. When investigating the silicated structures these substances have to be removed. Fossils only contain inorganic material.

1.2 Life form

Although some species form colonies, there is no differentiation within these. True mulitcellular development in plants requires walls that are strong enough to withstand the environmental forces they are exposed to and on the other hand the cell walls they have to be flexible enough to remain in close association during growth. Silicated structures don't have this properties.

The colonies are held together by interlocking siliceous structures; by pads or stalks of mucilage or by threads of polysaccharide that include cells within mucilage tubes, envelopes or sheaths.

1.3 Vegetative mulitplication and cell size reduction

Vegetative multiplication means that a cell undergoes mitosis and cytokinesis. The parental cell divides into two genetically identical daughter cells.

The mulitpartite cell wall shows a high degree of order. A major disadvantage of having silicated structures is that their shape does not change after formation. For cell division each daughter cell gets one valve from the origin cell and has to build a new one. The older valve is called epitheca, while the newer valve is the hypotheca. Each valve is associated with a set of girdle elements (cingulum), referred to as the epicingulum and the hypocingulum. New parts of the wall are formed within the protoplast and are then added to the wall by a form of exocytosis.

The new valves and girdle elements are smaller then the older parts of the cell wall because the formation of a valve takes place within the older valve. After cell division one daughter cell decreases in size compared to the mother cell, and one cell stays the same size. The hypotheca underlaps the edge of the epitheca. After mitoses and cytokinesis the hypocingulum becomes one daughter cell's epicingulum and the parental epicingulum becomes the epicingulum of the other daughter cell. Once incorporated in an epitheca, a cingullum will never be added to. This is because it is impossible to transport new material by exocytosis to the parts of the cell that lie outside of other silicated structures.

1.4 Sexual reproduction

During vegetative mulitplication the cells become smaller, so that the original size must be restored after various generations by so-called auxospore formation. Before that the cell's nucleus has to undergo meiosis in which the genome is reduced from haplwoid to diploid. Sexuality in diatoms, where it occurs, is always linked to the restoration of maximum cell size, which is achieved by the swelling of a specialised zygotic cell called auxospore. Meiosis can result in gametes that only contain half of the chromosomes compared to the diatom cell. After releasing the gametes the large non-motile female ones are fertilised by small, motile flagellate male sperm. The zygote (the result of the fertilisation, a diploid cell formed by two haploid cells) increases in size and forms an auxospore. Auxospores form new diatoms with restored size and new gene recombinations in their nucleus, which is the basis of evolution.

We don't know much about sexual reproduction in diatoms and the different forms that occur. It was studied in only a few species.[1]

1.5 Ecology

Diatoms can be found in all waters except the hottest and most hypersaline. They are abundant in marine and fresh waters all over the world.

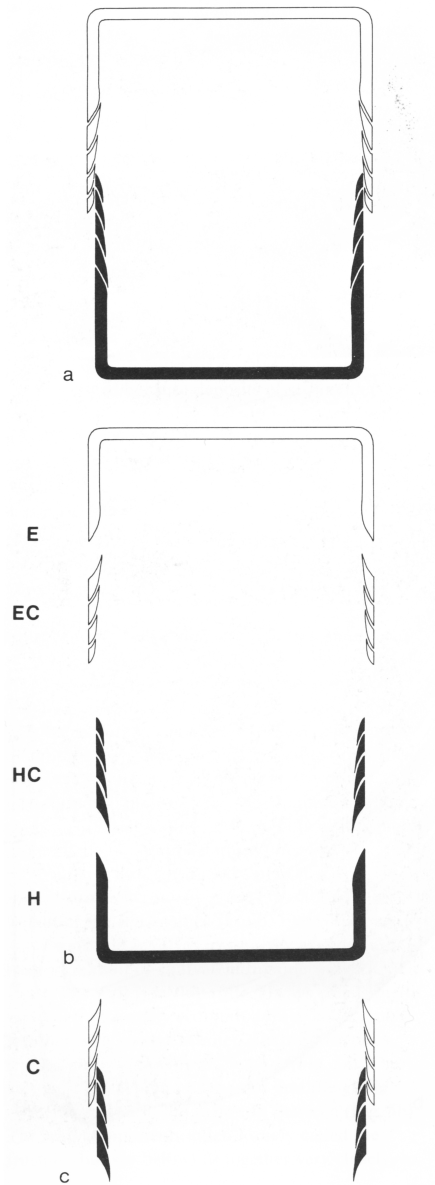

Figure 1: The components of a frustule. (a) A frustule in section, consisting of 2 thecae: an epitheca (white) and a hypotheca (black). The upper surface of the valve is termed the valve face and the downturned part, the valve mantle. (b) Expanded frustule: the epitheca consists of the epivalve (E) and several copulae, which together constitute the epicingulum (EC); the hypotheca likewise consists of hypovalve (H) and hypocingulum (HC). (c) The 2 cingula together are collectively referred to a the cinture (C) or girdle. With friendly permission of Dr. Richard M. Crawford and Dr. David G. Mann[1]

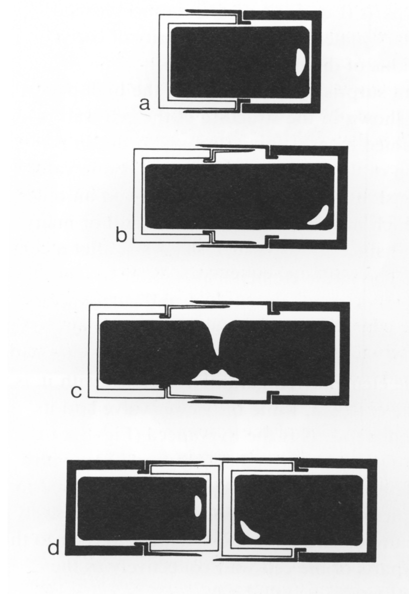

Figure 2: Growth of the cell during the cell cycle. (a) Newly released cell with complete epitheca and hypovalve (in many diatoms some components of the hypocingulum are already present at this stage). (b) Uniaxial growth of the cell by sliding apart of epi- and hypotheca, accompanied by addition of elements to the hypocingulum. (c) Completion of the thecae: mitosis and cytokinesis. (d) Daughter cells beginning to separate: new hypovalves formed beneath the girdle of the parent cell, i.e. beneath the epicingula of the daughter cells. With friendly permission of Dr. Richard M. Crawford and Dr. David G. Mann[1]

There are two influences on the growth and behaviour of planktonic diatoms: the availability of silicate (which they need for their cell walls) and the tendency for diatom cells to sink (as a result of the high density of their siliceous walls).

Turbulences in the water caused by wind, currents and convection often prevent the cells from sinking into deeper regions. Diatoms can deplete the silicate to very low concentrations. In the open ocean, where the level of silicate is often extremely low, the growth of diatoms depends on the transport from silicate-rich deep water. When conditions turn unfavorable, e.g. not enough nutrients or light available, the diatoms' sinking rate increases and the organisms leave the upper layer. Sinking diatoms often accumulate near the thermocline, a layer in the water where temperature rapidly changes with depth. Diatom cells in these resting populations re-enter the upper mixed layer when vertical mixing entrains them.

1.6 Influences on sinking velocity

1) In viscous situations the tendency of a particle to sink , expressed by its weight, increases with the cube of the linear dimension while the surface drag resisting sinking increases only as the square. This is presumably part of he explanation why the summer diatom plankton in dimictic[1] or warm monomictic[2] lakes is often composed of relatively small-celled centric species. [4]

   When the water is mixed turbulently, larger cells are at less of a disadvantage.

2) High sinking rates minimise concentration gradients of nutrients between cell and environment. This may select for shapes and sizes of plankton that have optimized sinking rates. [1]

3) To lower the density of the cell, heavy ions, e.g. calcium and magnesium, can be replaced by lighter ones, e.g. sodium or potassium. This is ineffecual in freshwaters because here not enough ions are available.

4) The shape of the planktonic unit (cell or colony) influences its sinking velocity. Changes of the shape that increase the surface area to volume ratio, e.g. cylindric shape compared to spheric symmetry, will increase the viscous drag on the cell and slow its descent.

Some shapes are adaptions to improve the efficiency with which cells are entrained within water movements. These and many other parameters decide which species will be present and when.

[1]Dimictic lakes mix from top to bottom during two mixing periods each year.
[2]Monomictic lakes mix from top to bottom during one mixing period each year.

2 *Corethron criophilum*

Corethron criophilum is a common marine centric diatom which can easily be found in Antarctic waters. The vegetative cell of *Corethron* is a cylinder of girdle bands with hemispherical valves at either end.

This diatom has a circlet of long spines on each valve, which causes the cells to form flocs in the water. At one end the long spines point obliquely away from the cell. The long spines on the other valve point in the same direction but not at so steep an gangle over the girdle region. In addition to the long spines on each valve, one of the valves has a series of shorter, clawed or hooked spines, that are directed away from the cell to form a crown.

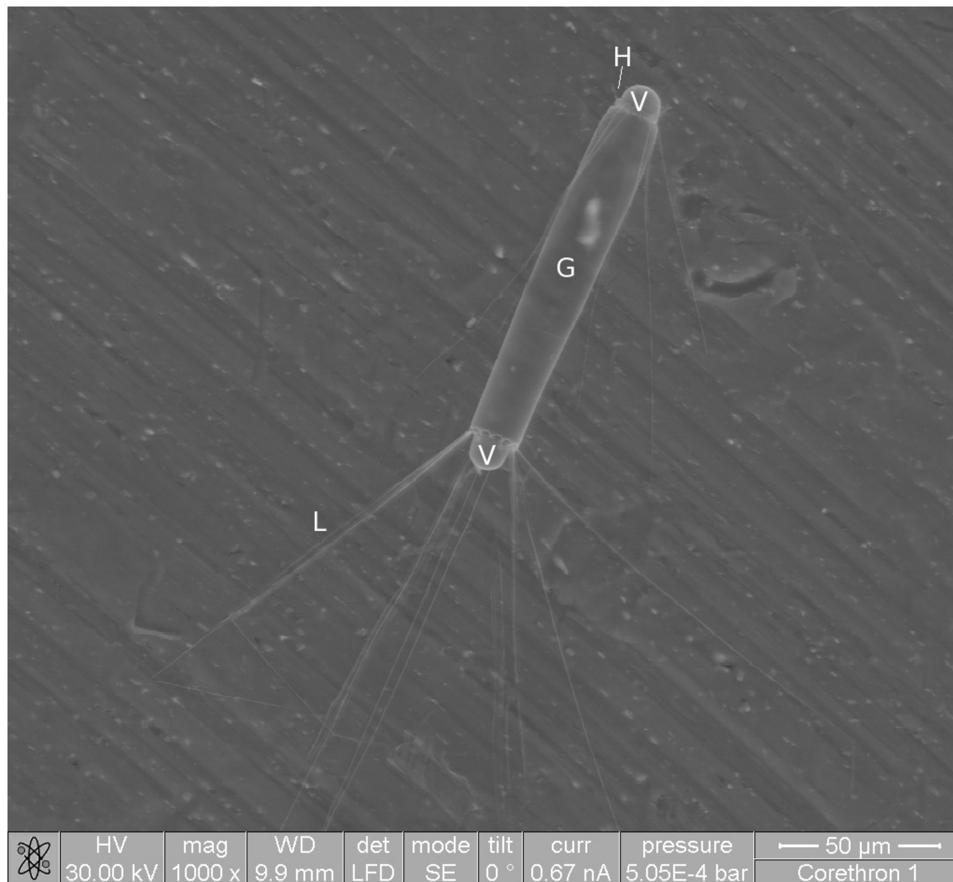

Figure 3: A cell of *Corethron criophilum* imaged with the ESEM. L...long spine, V...valves, H...hooked spine, G...girdle band

The different types of spines articulate with the valve(Figure 4). Crawford and Hinz [2] posed two questions concerning these spines:

- What is their structure, orientation and spatial relationship?

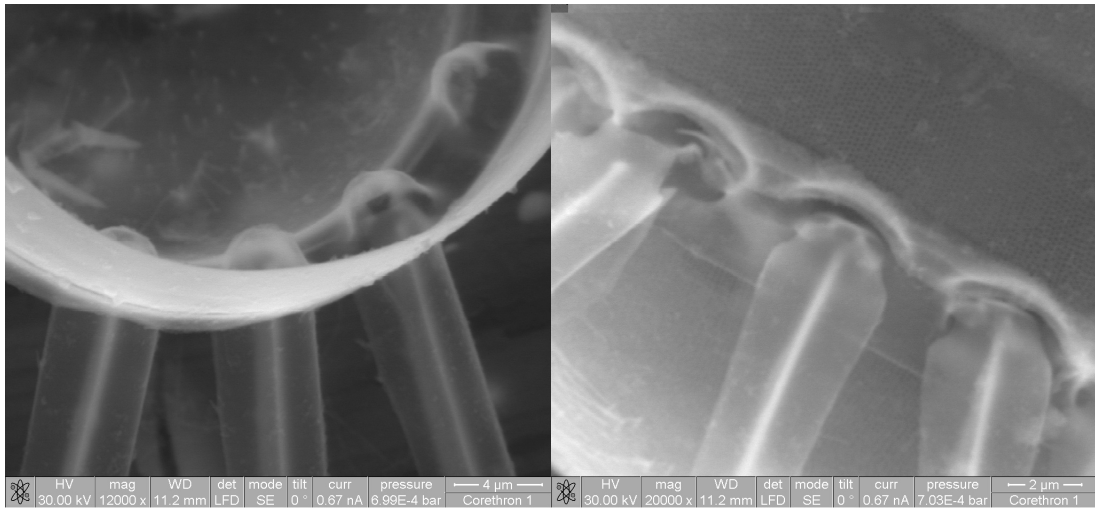

Figure 4: Articulated spines

- How important are they for the biology of the cell and population?

The long spines have tiny barbes that show an orientation which keeps cells apart (Figure 5). This could be of importance for a photosynthetic organism that depends on light. The forming of chains with other diatoms increases the chance of sinking out of the photic zone (surface zone of the sea or a lake having sufficient light penetration for photosynthesis).

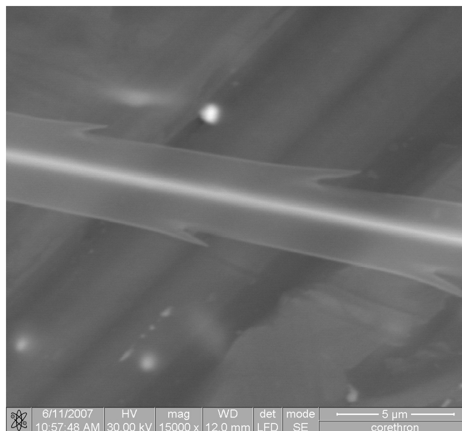

Figure 5: Detail of spine

2.1 Spines and vegetative cell division

Cells about to divide are recognisable by their increased length and by the position of the nucleus. It moves from the center of the cell to one side. After mitosis, during which the nucleus is divided into two genetically identical nuclei, cytokinesis starts: cytoplasm and the two nuclei are separated so that each end of the diatom will become a new cell.

The length of the parental cell wall doesn't change but the daughter cells contract slightly. A new valve with spines has to be formed for each daughter cell. During their formation the spines of each new-formed hypotheca show a parallel orientation at the center of the parental frustule. The girdle protects the dividing cytoplasm and the developing spines and controls their release when mature.

A lenghtening of the daughter cells follows but the distance between them remains constant. This withdraws the cylinders of the two cingula (the parental cell's former hypocingulum and epicingulum) away from each other and releases the spines. They can now move out from the line of the cylinder and swing out to adopt their final position. "...they move past a click-stop that prevents them moving too far back from their required position".[3] It is not clear how this is triggered and wether the movement of one spine type influences or even initiates the movement of another one. Crawford and Hinz suppose that "it is likely that the spines will be [...] no longer under the control of the cytoplasm. Consequently their movement outwards must be involuntary and a result of built-in instability".[2]

The hooked spines, of which left- and right-faced versions exist, are speculated to lock the long spines in place until the spines are allowed to swing out. It is thought that the newly formed spines are spring-loaded within their sockets and that if they were released too early, they would swing out and break the girdle bands. This would prevent further cell divisions.

The folding out of the spines moves the two daughter cells apart and in many cases they are separated.

3 Preparation

3.1 Sample Preparation

The probe we were dealing with was suspended in formalin for better conservation. This substance interferes with the examination especially when it dries under the microscope. The formalin seems to crystalise so that it is difficult to see the diatoms. It is also necessary to avoid debris that could disturb the micromanipulation. We got rid of the formalin by washing it out with distilled water. Therefor we used the centrifuge constructed by Clemens Grünberger (Fig. 6).

Figure 6: Centrifuge designed by Clemens Grünberger.

We used a micropipette to take 20 - 40μl of the sample suspension and dilute it with bidestilled water. Formalin has a lower density and also because of its nonpolar character it forms a second phase above the polar water. By spinning in the centrifuge we separated the denser diatoms from the formalin. It took us some time to find out (by trail and error) which rotation frequency to choose. If it is too low the diatoms with less weight stay in the upper formalin phase. If the forces are too high the shells might be destroyed and expecially the spines we were interested in. We obtained the best results with: 2000U/min[3]

After centrifugating for about 7 - 10 minutes we tipped off the supernatant with a pasteur pipette and diluted the remaining suspension with bidestilled water again. This procedure was repeated 4 or 5 times. The diatoms were settled as a pellet in the bottom of the tube. One should always keep the removed supernatant to ensure not to lose too much sample material if something goes wrong.

[3]Radius: 5cm

4 Light-microscopy

4.1 Principle of Inverted Reflected Light-microscopy

The main feature of an inverted optical microscope, compared to a conventional optical microscope, is that the objectives and turret are below the stage pointing up and the light source and condenser are on the top above the stage pointing down. The condenser and the additional light source above are necessary for transmission microscopy but during micromanipution these objects are displaced, because the space is needed for the micro-manipulator. So a design of combination of reflected light- and inverted microscope, called Le Chatelier design, would best suit our purpose. On the inverted stand, the sample is placed on the stage(Fig. 8). With this design the diatoms can be easily examined during micromanipulation. Reflected light microscopy is the method of choice for imaging specimen, that are not diaphanous[4] like most metals, many polymers and so on.

4.2 Contrast technique

Dark field and Bright field has different types of illumination, which results in differences in contrast. Dark field microscopy is a very common technique for samples that are not easily imaged against light background like diatoms, small aquatic organisms and many other biological samples. Dark field, in comparison to the Bright field, uses a Patch stop for blocking the center of the beam of light to produce a hollow cone of light. This cone allows the light to pass through the sample at oblique angles. Just the reflected or scattered light enters the objective lens and is seen as an image in dark field. The third available contrast technique was Differential Interference Contrast. DIC, like phase contrast, transforms the phase shift of light, induced by the specimen refractive index, into detectable amplitude differences. DIC enhances the contrast of unstained, transparent sample, so it is very useful for imaging such samples. We obtained best results in the dark field mode.(Figure 7)

Figure 7: Comparison between Contrast Techniques, Dark field(left), DIC(middle), Bright field(right)

[4]almost completely transparent or translucent

4.3 Investigation with an inverted microscope

The available glass slides turned out to be too thick for 500x magnification because we could not focus on the sample on the upper side. A possible solution is to turn upside down the slides so that the probe is closer to the light source. This can be done for optical analysis without manipulation. To enable micromanipulation from above we put a small amount of our prepared sample on the upper side of a cover glass that was placed over the light beam of the microscope. The inverted optical micrsoscope used for our studies is a Zeiss Axiovert MAT 200 (Zeiss, Germany)

5 Micromanipulation

5.1 Tip for micromanipulation

5.1.1 Basics of electrochemical etching

When a metallic electrode is dipped into a electrolytic liquid there is a concentration gradient between these two substances. This results in diffusion of metallic kations into the electrolyte which causes a difference of potential that counteracts the diffusion process. If voltage is impressed, so that the electrode has a positive potential compared to the electrolyte, the tendency of the metal to go into solution is increased.

5.1.2 Preparation of a tungsten tip

We used a high-concentrated solution of potassium hydroxid (KOH) as electrolytic liquid. The tungsten wire is not dipped into the electrolyte but the liquid is in contact with the wire at a ringlike section. W^{6+}-Ions are created by the applied voltage and anode-material is removed by the following chemical reaction: $W^{6+} + 6NaOH \rightarrow W(OH)_6 + 6Na^+$

The end of the wire is pulled down by gravity so that the region where the material is etched elongates. The ringlike etching zone and the tractive force caused by the weight of the wire's end finally leads to a good tip which can be used for micromanipulation.

5.2 Micromanipulation of the spines

We fixed the micromanipulator on the microscope table and approached the etched tip to the diatom of interest. To ensure that the tip only moves in the level directly above the cover glass we used the micrometer screw of the microscope table for shifting the diatoms instead of the tip.(Fig. 8)

By moving the spines towards the tip we tried to figure out how often they return to their former position when they are deflected. We started with little deflection to avoid breaking the spines. Then we moved the diatom away from to tip to see whether the new position is stable. The etched tip can easily be cleaned with aceton when too much material is sticking to it.

5.3 Discussion about the Micromanipulation-Movies

5.3.1 Elastic behavior of the articular

At the beginning of the Movie *Foxtrott*_05 elastic behavior of the hinge can be observed. After pushing the tail of spine in the direction of the valve, suddenly the spine snapps back into its old position and is kept (Figure 9). This elasticity in both directions(from and to the valve) of the hinge can be shown in the Movies *Echo*_03 and *Echo*_04.

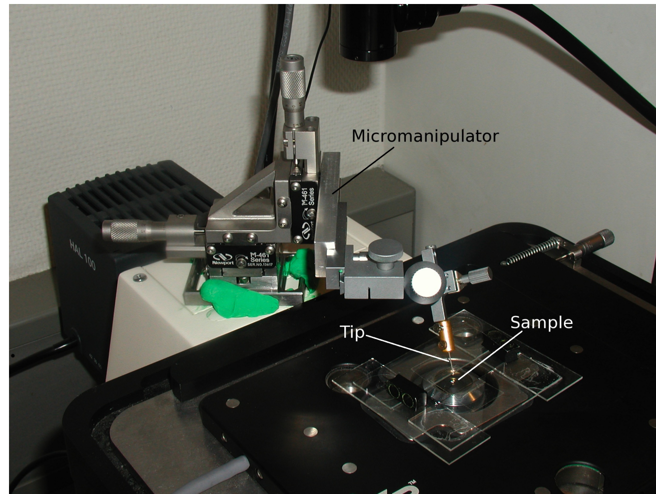

Figure 8: Construction of the Measurement facility

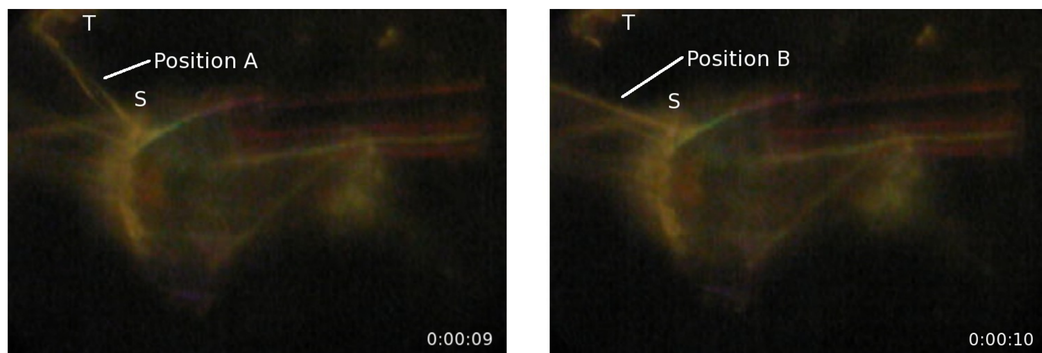

Figure 9: Articulated spines, T...Tip, S...Spine

5.3.2 Elastic behavior of the spines

In some cases the spines bend, when pressing the tail to the articular of the spine(Figure 11). This indicates the elasticity of the spines, which is necessary to prevent damage from turbulences in water or other disturbances.

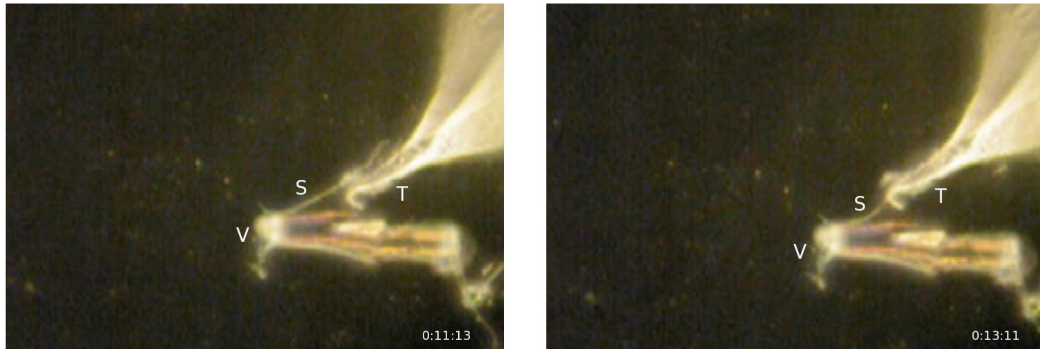

Figure 10: Elastic spines, T...Tip, S...Spine, V...Valve

5.3.3 Click-stop Mechanism

Manipulation of the *Corethron* in the video *Hotel* gave us a hint of the click-stop mechanism. We changed the orientation of the spine to the direction away from the valve by pushing it with the tip. After then the spine seemed to be in its final position because its kept this position and the hinge showed elastic characteristics. There are two possibilities:

- The spine snaps in the hinge through the click-stop mechanism.
- During the pushing process the spine can leaves the hinge and sticks to the glass slide.

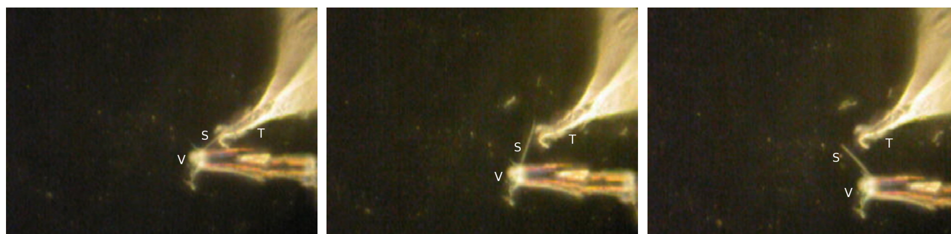

Figure 11: Elastic spines, T...Tip, S...Spine, V...Valve

5.4 Problems of Micromanipulation

These are challenging experiments that need patience, an of luck:

- Some spines, especially long ones, were attached to the glass slides. When trying to move these spines they broke.
- Many Corethrons were already damaged and fragile. Their spines and valves broke easily.
- Resolution of the camera for filming is too low to see details of the articular movement.
- Sometimes we obtained many *Fragilariopsis*. This Diatoms can obscure the *Corethrons*.(Figure 12)

Suggestions for future measurements:

- take camera with better resolution
- take video camera for longer records
- better centrifuge process

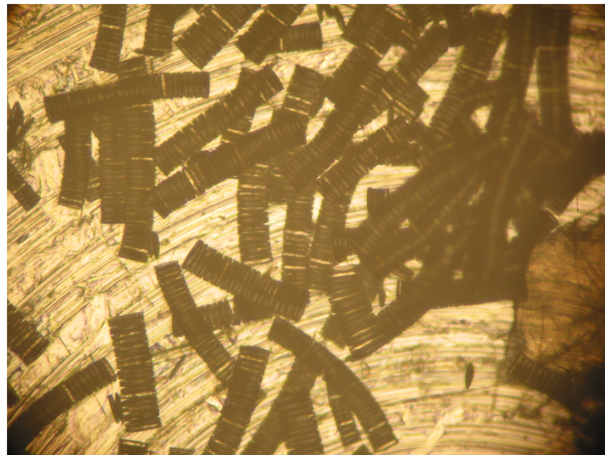

Figure 12: *Fragilariopsis* Diatoms

6 Electron-microscopy

6.1 Enviromental Scanning Electron Microscope (ESEM)

One cannot use a scanning electron microscope(SEM) to investigate samples that produce vapor when placed in vacuum. Usually biological samples must be dried or coated with gold or carbon to make biological material conductive. Using low-vacuum environmental SEM(ESEM) is more comfortable for diatoms frustules and avoids sample preparation.

Similar to a conventional SEM an electron beam whose energy ranges from 200eV to 30keV, is focused to a very fine beam and because of the interaction between this primary electron beam and the sample secondary electrons are emitted. This beam can be horizontally and vertically deflected by an objective lense, so it is possible to scan the sample surface in a raster mode.

In opposite to the high vacuum chamber of conventional SEMs, in the ESEM the sample is observed in a low pressure gaseous environment(ambient pressure is 130-2600 Pa). Water vapor or any other auxiliary gases are usable as a imaging gases. The secondary electrons accelerate to the detector, because of a voltage between sample and detector. After the collision with an electron, the gas atom ionizes if the energy of the electron is high enough. This process produces new unbound electrons, which accelerate too and this process is leading to a cascade. The water vapor functions as a cascade amplifier, amplifying the original secondary electron signal from the sample. The amplified secondary electron signal is detected at the gaseous secondary electron detector. During scanning the sample, the detector gets a intensity for every point(x,y) on the sample, which is used to image it into a brighter or darker portion.

In conventional SEM nonconductive samples are charged due to the primary electron beam and produce distorted images. But in ESEM the positive charged ionized water vapor molecules move into the opposite direction to the sample and thereby neutralize the negative charge of the sample surface.(Figure 13)

ESEM is a good alternative technology for high resolution imaging without coating or other destructive preparation techniques. But for good imaging the right combination of accelerating voltage, spot size, vapor pressure and working distance has to be set, which is challenging.

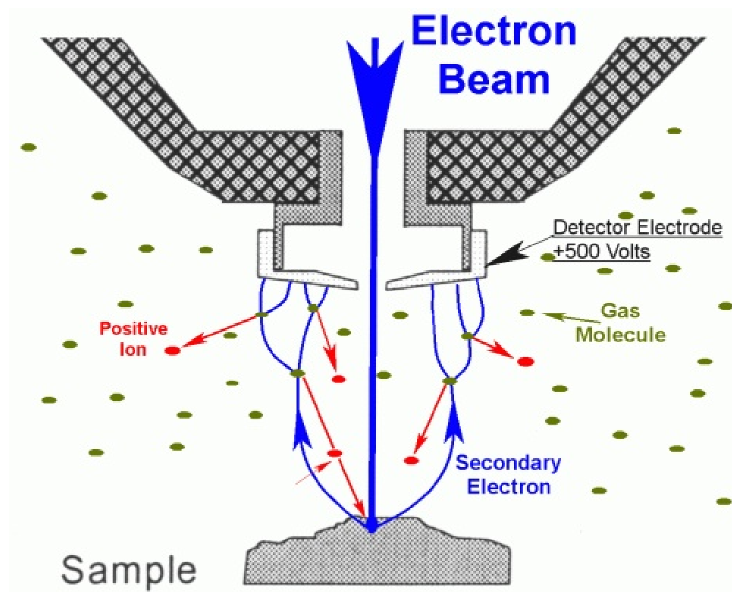

Figure 13: Principle of ESEM [6]

7 Attachment

7.1 Equipment

- Preparation
  - Pipette: Eppendorf Research 200
  - Tips: epT.I.P.S. Standard 2-200mikrol
  - Poly-L-Lysine Solution
  - micro slides, cleaned: 76 x 26mm
  - cover glasses: 18x18mm
  - centrifuge-motor
- Micro-manipulation
  - Micro-manipulator: Newport M-461
  - Tips: epT.I.P.S. Standard 2-200mikrol
  - Inverse-microscope: Zeiss Axiovert 200 MAT
  - Camera: Canon Power Shot G2
- Electron-microscope: Company FEI, Quanta 200 3D

7.2 Documentation about the movies

Corethron	Video	Nothing	Spines			Comment
			damaged	snap back	stayed	
Alpha	1	x				big Tip[5]
	2		one			big Tip
	3	x				big Tip
	4	x				big Tip
	5	x				big Tip
	6	x				
	7	x				
	8		one			
	9	x				
Bravo	1		two			
	2	x				
	3		four			
	4		two			
Charlie	1	x				
	2	x				
	3	x				
Delta	1	x				underwater, difficult to see
	2				X	elastic spine
	3				X	elastic spine
	4				X	elastic spine
	5	x				
	6				X	elastic spine, wedged with *Fragilariopsis* [6]
	7				X	elastic spine, wedged with *Fragilariopsis*
	8	x				
	9				X	elastic spine
	10				X	elastic spine
	11				X	elastic spine
	12	x				
	13				X	elastic spine, wedged with *Fragilariopsis*
	14				X	elastic spine, wedged with *Fragilariopsis*
	15	x				
Echo	1	x				
	2		one			
	3			X		stiff spine, elastic articular

[5] We used two types of tips, a big tip and a hook tip. If it is no comment about the tip written in the column, the hook tip was used.

[6] Another diatom species, which occurs often in the sample. These diatoms are heavier than the *Corethron*.

Corethron	Video	Nothing	Spines			Comment
			damaged	snap back	stayed	
	4			X		stiff spine, elastic articular
	5			X		stiff spine, elastic articular
	6		one			
	7	x				
Foxtrott	1				X	stiff spine
	2			X	X	stiff spine
	3			X		
	4			X		stiff spine
	5			X	X	stiff spine, very interesting
	6				X	jumped away
	7			X		moved back
	8				X	
	9	x				
	10			X	X	
	11		one			
	12			X		
	13			X		
	14	x				
	15	x				
	16	x				
	17		one			
	18	x				
	19		three			
Golf	1	x				erasing[7]
	2		one			
	3	x				erasing and elastic spine
	4	x				elastic spine
	5		one			
	6		?			
	7		one			spine jumped away
	8		one			
	9		one			
	10		one			
Hotel	1		three			
	2	x				
	3					elastic spine
	4				X	elastic spine
	5					elastic spine, maybe click-stop snap in or
	6				X	
	7					damaged or snapped in
	8			X	X	

[7]"erasing" means destruction of the spine without moving it.

Corethron	Video	Nothing	Spines damaged	Spines snap back	Spines stayed	Comment
	9			X		seems to be in the final position
	10					elastic spine
	11					elastic spine
	12				X	
	13				X	
	14		X		X	
India	1	x	two			The damaged wasn't recorded
	2			X		erasing and elastic spine
	3	x				
	4			X		
	5	x				
	6	x				
	7			X		
	8				X	
	9	x	one			
	10		one			
	11					
	12					
Juliett	1				X	very elastic spine
	2				X	erasing too
	3			X		
	4			X	X	interesting
	5			X		erasing
	6			X	X	
	7			X	X	
	8		one		X	
	9		three		X	
Kilo	1	x	one			
	2	x	one			
	3	x	one			
Lima	1	x				erasing
	2				X	
	3	x	one			
	4	x				
	5	x				erasing
	6		one			
	7	x				
	9			X		
	10		one	X		
	11		one		X	
	12		one	X		
	13		two			
	14	x				erasing
	15	x				

Corethron	Video	Nothing	Spines			Comment
			damaged	snap back	stayed	
	16	x				erasing
	17				X	
	18	x				
	19			X		
	20			X		
	21			X		
	22			X	X	
	23			X		
	24			X	X	
	25		one			
	26	x				
	27			X	X	
	28				X	
	29	x				
	30	x				
	31		one			
	32		one			
	33	x				
	34	x				
	35				X	
Mike	1	x				erasing
	2	x				erasing
	3	x				
	4				X	elastic
	5	x				
	6			X	X	
	7			X	X	
	8	x				erasing
	9	x				
	10	x				
	11	x	two			
	12	x	one			
November	1	x				
	2				X	
	3				X	
	4		one			
	5			X	X	
Oscar	1	x				erasing
	2			X		disappeared suddenly
	3				X	
	4	x				
	5		two			
	6	one				
	7					bended spine
	8		X			
	9	x				

Corethron	Video	Nothing	Spines damaged	Spines snap back	Spines stayed	Comment
	10		one			
Papa	1	x				erasing
	2		one			
	3		one		X	
	4		one		X	
	5	x	one			

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