DIPLOMARBEIT

Atomic Force Microscopy of *Bacillus subtilis*

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durch

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Abbreviations

AC	Alternating Contact
AFM	Atomic Force Microscope/ Microscopy
ASTM	American Society for Testing and Materials
CNS	Central Nervous System
DC	Direct Contact
DLC	Deflection Lateral Correction
DNA	Desoxyribonucleic Acid
DVD	Digital Versatile Disc
GMO	Genetically Modified Organism
HD	High Definition
HOPG	Highly Orientated Pyrolytic Graphite
LD	Light Source Diode
LVDT	Linear Variable Differential Transformer
MEMS	Micro-Electro-Mechanical-System
NEMS	Nano-Electro-Mechanical-System
OECD	Organization for Economic Cooperation and Development
PBS	Phosphate Buffer Solution
PD	Photodiode
SAPS	Small Acid Soluble Proteins
SAM	Self Assembling Monolayer
STM	Scanning Tunneling Microscope/ Microscopy
TEM	Transmission Electron Microscope
TSB	Tryptic Soy Broth

Abstract

Nanotechnology and especially nanobiotechnology are continuously growing fields. The investigation of living samples and biological processes provides new approaches for developing applications and devices in biology and health care. Working on a nanobiotechnological level is a very delicate issue, considering imaging and sample preparation techniques and one will soon reach the technical limits of nanotechnological investigation methods.

Within this thesis the Atomic Force Microscope proved to be a powerful tool for nanobiotechnological investigations on *Bacillus subtilis*. *Bacillus subtilis* is a single- celled bacterium, which has the ability to sporulate and thereby survives extreme environmental conditions. *Bacillius subtilis* is not harmful to human health and its spores, which are extremely resistant to environmental influences, serve as safe model organisms for pathogenic microorganisms in water hygiene. *Bacillus subtilis* is used to evaluate water disinfection devices using ultraviolet radiation. By inducing adverse environmental conditions to living *Bacillus subtilis* cells the sporulation process is successfully initiated and the different morphological stages are - for the first time ever - imaged using AFM techniques. Two different types of spores due to the different methods of inducing the sporulation are investigated. One type of spores is resistant to UV-radiation whereas the other type is very sensitive to UV-radiation. The different types are investigated by Force Mapping techniques using the AFM and the cell surface properties are finally compared. Our investigations revealed that UV-sensitive *Bacillus subtilis* spores are softer when measuring the indentation depth of the cantilever tip at a preset trigger force whereas the UV-resistant spores show a lower indentation depth compared to the UV-sensitive spores using the same force.

1 Nanotechnology

1.1 Introduction

“There´s plenty of room at the bottom”[1], with this sentence the Nobel Laureate Richard P. Feynman[1] gave the starting signal for a new era of technology and the idea of small became a new definition. During his speech on December 29, 1959 at the "Winter Meeting of the American Physical Society" at the California Institute of Technology, Feynman proclaimed the idea of manipulating and controlling things on a small scale. “Why cannot we write the entire 24 volumes of the Encyclopaedia Britannica on the head of a pin?”[1], his idea bringing information down to a small scale and have therefore more storage space, the thought to understand the interaction between biological systems, ”biology is not simply writing information; it is doing something about it”[1]. He had the vision of miniaturizing the computer and the production of tiny machines. Despite all these future prospectives Feynman also touched upon the problems of working in a very small world, “The principles of physics, as far as I can see, do not speak against possibilities of manoeuvring things atom by atom. It is not an attempt to violate any laws; it is something, in principle, that can be done; but in practice, it has not been done, because we are too big”[1]. The small world down to atomic and subatomic resolution has to satisfy the laws of quantum mechanics and therefore the behaviour is different compared to things on a large scale[2].

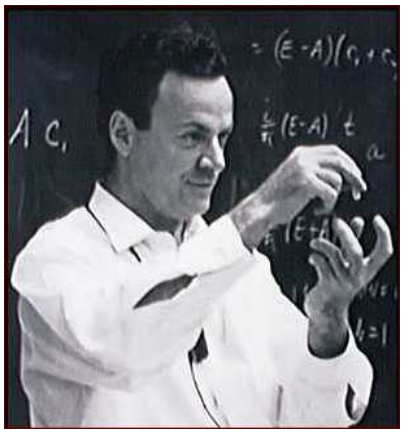


Figure 1: Richard P. Feynman[3].

[1] Richard P. Feynman, 1918-1988, theoretical physicist,
Obtained the Nobel prize for the theory of quantum electrodynamics, which governs every physical and chemical process except those embracing gravitation and radioactivity.

1.2 Definition

Nanotechnology can be defined as research and technology performed at the length scale of 1-100nm, which has its application in the real world. Nanotechnology combines the application, the production of systems and the scientific research as well as the integration of molecules or atoms into larger systems in the field of physics, biology, chemistry and medicine. It is the planned manipulation of materials and properties on a nanoscale, considers the understanding of the interaction between nanostructures, their properties and how these can be engineered, characterizes materials on the nanoscale and enables the control of size and the manipulation of nanoscale structures[4].

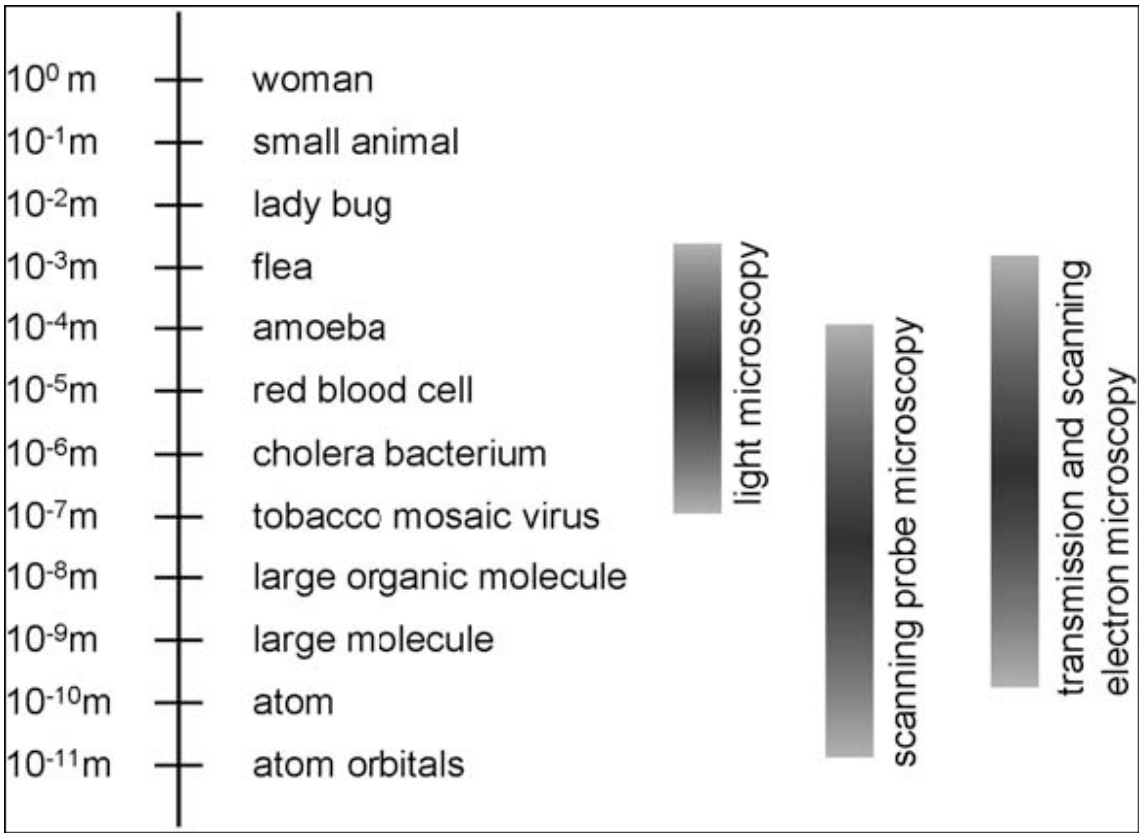


Figure 2: Scaling of the microcosms and the relevant microscopically techniques[5].

1.3 Motivation

Research at the nanoscale provides various unique applications and opens a new field of sciences and technologies. There are a lot of opportunities to discover new properties of materials and processes, as well as the development of new nanostructured materials and quantum mechanical laws[2] and therefore new behavior of materials and systems. The goal is to miniaturize applications used in electronics as much as possible, i.e. in the computer chip

industry, to create new materials, which have their application in everyday life and to understand the mechanisms of biology to work with the smallest components of life. The development of medical systems and machines for health care has become increasingly important in the last years. Biological sensors have been developed, which operate at the quantum limit of sensitivity systems.

According to M. Roco, a senior advisor on nanotechnology to the US National Science Foundation, there are four generations[6] (see Figure 3) of nanotechnology. The first generation is the period of passive nanostructures, i.e. the development of materials on the nanoscale performing one task. The second phase represents active nanostructures, which are multitasking and interacting with other devices, such as sensors and actuators. In the third generation of nanotechnology the combination of many nanocomponents to a nanosystem will be found. The fourth phase will be the era of molecular nanosystems, which will be developed by the combination of biotechnology and nanotechnology and provide a lot of benefits in health care and environment safety[7].

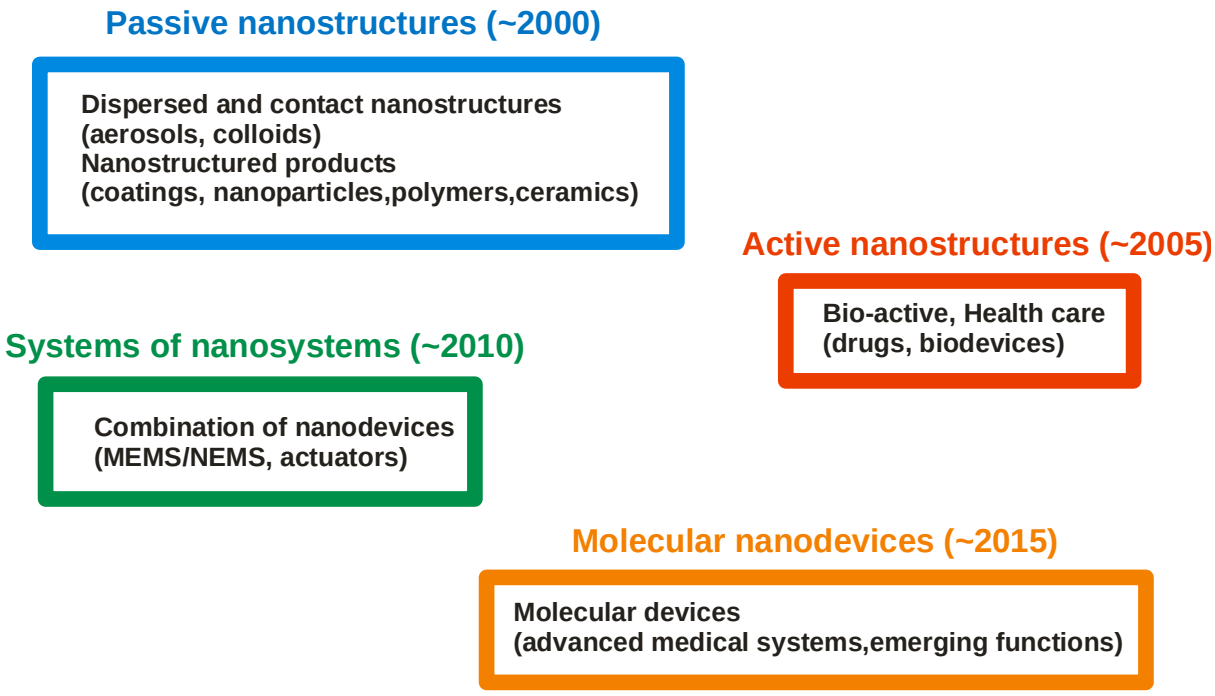


Figure 3: The four generations of nanotechnology.

1.4 Tools and Applications

Nanotechnology and its applications can be divided into different fields of interest.

Nanomaterials

This research field deals with the study of materials, their production, the understanding and their behaviour on a nanoscale. The investigation of new structures and the manipulation of materials offers many new properties, not only in industry but also in medicine[2], i.e. carbon-nanotubes, which are very interesting for medical treatment, because of their mechanical strength and their ability to bind specific antibodies. The usage of nanocrystals, because of their optical and electrical properties, and nanoparticles has become a very interesting field of research. For example, a quantum dot is a semiconductor nanostructure (InGaAs, CdSe)[8], that has already found applications in many consumer electronics devices. The new HD-DVD player and blue ray discs need all the use of a blue laser for data reading. Years ago, the development seemed to be impossible, until the development of a blue quantum dot laser[9].

Figure 4: Fluorescence induced by UV-radiation of different sized CdSe quantum dots[10]. They confine the charge carrier in all three spatial directions, so that the spectrum is discrete depending on their size.

Research and development of nanomaterials is directed toward understanding and development of new materials, devices and systems, such as protective coatings, light weight materials, self–cleaning clothing and many more.

Nanoanalysis

This research field deals with the development of techniques and the investigation of structures of materials on the nanometer scale. There are many instruments that are used for investigating nanostructures and especially crystals. Gathering information from nanostructures is not very easy, because at this scale of size surface effects are increasingly

important and a lot of attention has to be paid on the choice of the measuring instrument, the sample preparation and the interpretation of the measuring results. Today many analysing instruments, such as AFM (Atomic Force Microscope), STM (Scanning Tunnelling Microscope), TEM (Transmission Electron Microscope) and many more are in use to analyse different materials.

Nanoelectronics

This field of research deals with the usage of nanotechnology on electronic systems. There is no clear definition of nanoelectronics, but the main field of nanoelectronics considers transistors and integrated circuits of about 100nm and below. The usage of nanowires, molecular and hybrid electronics are gradually replacing common electronic materials. These very complex electronic circuits with their integrated elements of about 100nm are produced by photolithographic methods. The so-called, "top-down" nanotechnology deals with the fabrication of nanoscale structures by using micro- and nano- lithography and etching methods. These methods are mainly used in the semiconductor industry for producing microprocessors. The main industrial sector of nanoelectronics is the computer market[11,12].

Nanooptics

Nanooptics focus on the research of nanostructured materials with new optical properties, on the development and the production of optical systems at the nanoscale. This includes the investigation of novel optical effects and the focus of nanooptics in optoelectronic components. Nanooptics is industrially found in equipments for electronic production (photolithography), telecommunications, multimedia (blue-ray discs), measuring equipments (sensors), and microscopes (mirrors and lenses)[13].

Molecular nanotechnology

The bottom-up or molecular nanotechnology deals with self-assembling structures of biological systems. Self-assembly is used by biological mechanisms to develop very complex, functional structures and systems. SAMs (self assembling monolayers) are one example that use molecular self-assembly to create structures and have their specific functions on the nanometer scale. SAMs are technologically very useful systems and provide very useful properties, i.e. formation of crystals, colloids, polymers and many more.

Today nanotechnology has become an ambitious science. There are many fields of application in industry and our daily life. For example a variety of MEMS/NEMS (Micro-Electro-Mecanical-Systems/Nano-Electro-Mecanical-Systems) have been produced which are commercially used. MEMS/NEMS are mechanical elements, which are integrated on a chip combined with sensors, actuators and electronic components. They are used for devices where sensors, actuators and electronic have to work together, i.e. accelerometer in airbags and or in cameras to avoid blurred images and many other applications[14], the detailed description of which would go beyond the scope of this work.

1.5 Risks

Although nanotechnology has brought many advantages to our life, these new technologies might bring up a lot of risks considering the preservation of health and the environment. It is not known which influence nanoparticles can have on human health and the environmental safety. Free nanoparticles could enter the human body by inhalation, ingestion or via the skin or simply disperse into the environment[15]. There has to be a differentiation between the unintentional effects to the body and the levels of exposure. Nanoparticles have the ability to affect cells by intruding into their cell membranes. Because of their size and shape they can deceive the immune system and therefore resist many defence mechanisms of the human body. Once integrated to the body they can probably harm the central nervous system (CNS), can penetrate the blood-brain barrier and could have toxic effects to the organs. For example, it is still not known if nanoparticles can pass from a pregnant woman's body via the placenta into the unborn child, although expectant mothers might use cosmetics modified with nanoparticles[16].

In the future there have to be many investigations considering the release of nanotechnological material to the environment and thus also to humans, animals and plants. Conventional testing and assessment methods have to be revised in order to focus on the different properties of nanoparticles and systems. It has to be considered that new approaches also have their possible risks and dangers of being abused.

1.6 Limitations

The limits to smallness are very clear if one considers the sizes of systems, devices and machines presented in the size of atoms and represented by molecular particles of biology.

Today with our current technology and know-how it is possible to develop machines and systems that are smaller than the diameter of a human hair and there have been experiments assembling atomic sized particles by using scanning tunnelling microscope. Currently it is possible for chemists to produce small molecules, but the challenge of nanotechnology is to characterise, develop and control the complex structures of molecular sized components[2].

1.7 Perspectives

Today nanotechnology has established to be a very promising field of scientific research and industry including mass-market consumer products, such as nano-coatings for self cleaning glass reacting windows to sunlight, smart clothes, wearable electronics, sports equipment, cosmetics, nanotubes for delivering drugs and many other innovations[9]. In the next years nanotechnology will play a huge role for many applications in our life. In electronics and communications, there will be a development of new flat-screens, data storage will be enlarged, processing speeds will be raised, power efficiencies will be improved, there will be new developments of chemicals and materials, such as smart magnetic fluids for vacuum seals and lubricants. In medicine and healthcare, new sensors for "labs-on-a-chip", new gene and drug delivery systems, bio-compatible body parts and fluids will be available. For environment saving there will be nanomechanical systems which can remove pollutants from industrial influence and increased opportunities for recycling. New camouflage materials, detectors of chemical and biological agents and hard nanostructured coatings will find their origin in nanotechnology. Despite all these promising innovation also the negative effects of nanotechnology have to be considered and controlled[17].

1.8 Biotechnology

Since 6000 years humans have used living cells and enzymes, e.g. bacteria in yoghurt, yeast in brewing and baking, rennet in cheese making, although they did not know what they were doing, until the French chemist, Louis Pasteur discovered in the 19th century that brewing, winemaking, and other kinds of fermentation involved specific microbes[12,18]. Since this point of time biotechnology has become a very interesting field of research. It has an enormous scientific and economic potential in health science, agriculture and environmental technologies. Like nanotechnology, biotechnology acts interdisciplinary. It is a combination of microbiology, virology, genetic engineering, zoology, botany, biochemistry and many

other sciences. To get a better understanding of the different scientific fields of biotechnology, the OECD suggested in 1989 separating this technology into three working fields, the classical, the modern and the molecular field. The basis of the classical biotechnology is the usage of the ubiquitary nature. The modern biotechnology deals with the investigation of new organisms and their usage, and molecular biotechnology is focused on genetic engineering, the analysis and mutation of genomes and genetic materials.
There are four subfields considering biotechnology. The specific applications are defined by the OECD by different colours, white, red, green and blue[19].

White biotechnology

This kind of biotechnology deals with industrial processes, the usage of organisms for chemicals and enzymes to synthesise products, which are easily degradable. Bacterial enzymes are used in food manufacturing and as active ingredients in washing powder.

Red biotechnology

The research field of red biotechnology considers medical processes. It deals with the development of drugs for medical use by genetically modifying yeast and bacteria.

Green biotechnology

This biotechnology has its applications in agricultural processes. There is a development of genetically modified organisms (GMO), which save the environment and have desired effects, such as the resistant to certain chemicals and pesticides.

Blue biotechnology

This part of biotechnology focuses on marine biology research. Because of the diversity of genomes found in waters, scientists hope to find new substances for medical treatment. There is also the study of sponges and diatoms which have necessary enzymes to produce silica structures and could be used for growing chips in a laboratory.

1.9 Nanobiotechnology

Nanobiotechnology is a combination of Nanotechnology and Biotechnology and has become very important in the last ten years. Nanobiotechnology is based on the cooperation of physicists, biologists, chemists, medical doctors and engineers[12]. It does not concentrate on a single atom, but is interested in certain functions and processes, i.e. genes as carrier of the

DNA, the connection of antibodies to debris, and other biological, chemical, physical functions, which provide the operability of organisms. Nanotechnology provides the instruments and processes to investigate biological and medical activities. The aim of nanobiotechnology is to understand the mechanisms, the structure, the organisation and the repair mechanisms of organisms. If we understand how this "socializing" of molecules works, a new way of new technologies is open. For example, it is possible to develop pharmaceutics that deliver drugs through the body and release them only at certain cancer cells without any adverse reactions for the rest of the body. Today scientists work on a new computing system on the basis of DNA. Such systems would be approximately a billion times more energy efficient and would have a 10^{12} times better processor performance[12].

Some examples of nanobiotechnological products, which have proved as very useful are briefly described.

Magnetic particles i.e. iron oxide directly injected into a tumour are contactlessly activated. When a moving magnetic field is applied, the particles start to vibrate. This vibration creates heat between 42°C and 70°C, which irreparably damages the tumour and healthy cells are left intact. Nanoshells are used for photo-thermal cancer treatment. They consist of a silica core covered by a thin gold shell. The size, shape and composition give them a unique optical property. Researchers can tailor nanoshells to respond to a specific wavelength of light. If externally energy is supplied to these shells, this energy is directly absorbed and an intense heat is created, so that the carcinogenic cells are killed. Carbon nanotubes are rolled-up sheets of carbon atoms and are extremely small. The detonation nanotubes are filled with water molecules. When the nanotubes are exposed to laser light, the water molecules vaporize, which produces enough pressure to blow up the tubes and any cancer cells in the immediate vicinity[20].

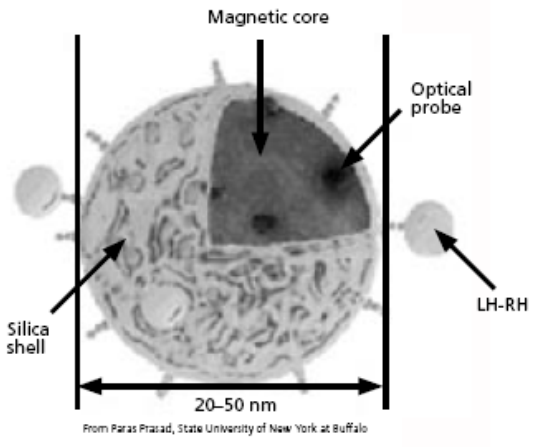


Figure 5: Multifunctional nanoparticle[21].

2 Bacillus subtilis

2.1 General information

Bacillus subtilis (lat. Bacillium/bacillus, stick; subtilis, simple) is a rod-shaped, gram-positive bacterium with flagella and the ability to sporulate. The bacillus is commonly found in soil. Like every bacterium of the species Bacillus, *Bacillus subtilis* grows in aerobe conditions and has the ability to form a tough, protective endospore, which allows the organism to resist extreme environmental conditions e.g. pH, temperature and nutrient shortage. The process of reversibly forming a tough and protective endospore that allows the organism to tolerate extreme environmental conditions is called sporulation[22].

Bacillus subtilis was discovered in 1835 by Christian Gottfried Ehrenberg and described as *Vibrio subtilis* (bent chopstick). The rod-shaped *Bacillus subtilis* is about 2-3µm in length and about 0.6µm in height. The several flagella arranged all around the bacillus offers it the ability to move forward. *Bacillus subtilis* is ubiquitous distributed and can be isolated from earth, grass and hay (therefore it is also called grass or hay bacillus), water and air. It habitats the rhizosphere and the superficial layers of the ground. It subsists on nutrients that are produced by other organisms. Because of its typical function as a decomposing bacterium it reverses organic substances in nature and has therefore a very important role within the nutrient circle. It is not considered a human pathogen[22,23]. Because of his phylogenetic relation to other pathogens, such as Listeria and Staphylokokken *Bacillus subtilis* is very popular for biological and medical investigations.

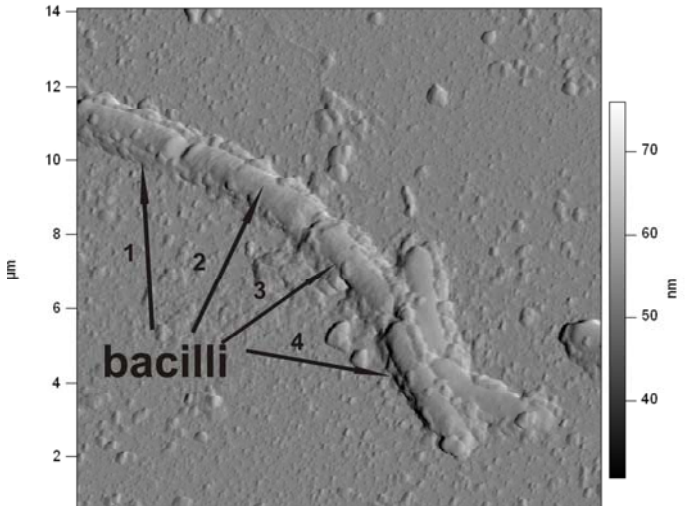


Figure 6: Chain of four vegetative *Bacilli subtilis*, Amplitude Trace, scan size 14µm x 14µm.

2.2 Applications

Today *Bacillus subtilis* is used for many applications. Because of its enormous heat resistance it is an indicator for many sterilization processes in medicine, pharmacy and food industry, i.e. *bacillus subtilis* serves as safe model organism for pathogenic microorganisms in drinking water[23]. In agriculture it is found as a fungicide for beans, corn, wheat, and many other plants. It acts as a biological control agent and some extra cellular enzymes which are produced by the bacillus are used as additives in laundry detergents. There are many medicaments containing cells or spores of *bacillus subtilis* for the treatment of gastro-intestinal diseases and cancer patients.

2.3 Morphogenesis

Bacillus subtilis can exist in two alternate states of living, the vegetative growing and the sporulation. Sporulation is a mechanism of bacteria to adapt to starvation. Unlike most adaptive responses in bacteria, sporulation takes many hours and includes major changes in cellular morphology as well as in biochemistry and physiology[24]. The first morphological characteristic of sporulation is the asymmetric division of the protoplasm. Protoplasm is the living substance inside cells with a cell membrane, considered as the smallest autonomous viable unit. Morphogenesis needs the cooperation of two sister cells, which both are starting with the same genome. Each part of the divided mother cell has a different function during the sporulation process. The smaller part ends in creating the spore, the bigger part is the mature spore contributing most of its sources and lyses in the end. In this stadium the spore is built in an internal double membrane-bound called fore spore. The fore spore is situated within the plasma of the mature spore and so the whole metabolism is depending on the nutrition of the mature spore[25]. Most of the nutrient resources during the sporulation process are required for building the thick and multilayered spore walls. For several hours protective structures assemble inside of and around the fore spore. The completed spore is encased in a multilayered protein shell known as the coat. The coat is the outermost layer of the spore. After the sporulation process the spore gets released and its metabolism is incapable of measurement, the mother cell lyses. The spore has only the task to survive the life threatening conditions. Spore dormancy and resistance depend on the partial dehydration of the interior compartment of the spore. Although there is no metabolism, the spore reacts to changes in the living conditions. If the living conditions are tolerable the process of germination begins.

When germination is triggered, water enters the spore core, which swells and the spore converts back into a vegetative cell[24].

2.4 Spore fundamentals

Bacterial spores are formed in response of starvation during a sporulation process of approximately 8 hours. They can resist almost any stress of nature for a very long period of time in their dormant state. Although the spores reside in a dormant state, they are sensitive to their surrounding and recognize if the living condition allow reconverting to an actively growing cell[26]. All *Bacillus subtilis* spores have a common structure (see Figure 7). In the center of the spore, there is the core or spore protoplast, which is a dry compartment containing the DNA, the cytoplasmic membrane, the cytoplasm, ribosomes, and many other cellular essentials. The core is surrounded by the inner membrane and the cortex, which is a thick layer of peptidoglycan (PGN). PGN is a macromolecule consisting of sugar and aminoacids, which provides the stiffness of the cell wall, also called murein. The cortex is surrounded by the coat, which is a complex protein shell. The coat has the function to protect the spore from chemicals, such as hydrogen peroxide, acts as an elastic material and has the ability to perform enzymatic reactions[25].

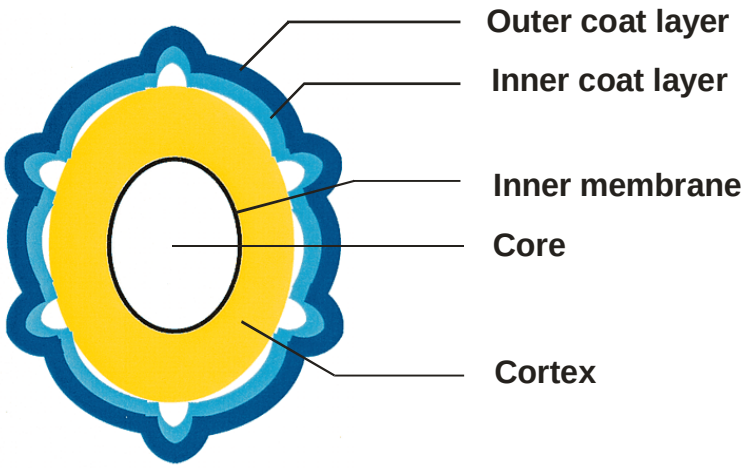


Figure 7: Cross section of a *Bacillus subtilis* spore[25].

Another characteristic of endospores is the content of dipicolinic acid, which is found in the spore cores but not in vegetative cells. The concentration of calcium ions is much higher in spores than in the living cells. The compound of calcium and dipicolinic acid, the so called

calcium-dipicolinic acid complex, has the function to reduce the water content of the endospore. About 10% of the endospore consists of the calcium-dipicolinic complex. The dipicolinic acid is responsible for the heat resistance and the calcium covers the spores from oxidizing agents. The high level of the calcium-dipicolinic complex reduces the water content of the cell during the sporulation, so that in the end the endospore contains only of 10-25% of water of the mature spore. The lower the water concentration the higher is the resistance of the spore to heat. Dehydration has also an influence to the resistance to chemicals[27]. Another difference to a vegetative cell is the pH of the core, it is more than one unit lower than the pH of the vegetative bacillus. During the sporulation process a high concentration of proteins is produced, the small acid-soluble proteins (SAPS), which bind to the DNA in the core. They are responsible for protecting the DNA from desiccation and radiation, especially UV radiation, which could lead to a mutation and denaturation. The SAPS also acts as a carbon and energy source for the germination. Endospores can be located centrally, terminally or subterminally within a cell. In most cases the endospore is much larger in diameter and so the cell seems to be swollen at the location of the endospore[26].

Differences between endospores and vegetative cells:

Characteristics	Vegetative cell	Endospore
Structure	Typical gram-positive cell A few gram-negative cells	Thick spore cortex Sporecoat
Microscopic appearance	Nonrefractile	Refractile
Calcium content	Low	High
Dipicolinic acid	Absent	Present
Enzymatic activity	High	Low
Metabolism (O_2 uptake)	High	Low or absent
Macromolecular synthesis	Present	Absent
mRNA	Present	Low or absent
Heat resistance	Low	High
Radiation resistance	Low	High
Resistance to chemicals and acids	Low	High
Stainability by dyes	Stainable	Stainable only with special methods
Action of lysozyme	Sensitive	Resistant
Water content	High, 80-90%	Low, 10-25% in core
Small acid soluble proteins	Absent	Present
Cytoplasmic pH	About pH 7	About pH 5.5-6.0

Table adapted from [26].

3 Sporulation

Sporulation is generally induced by starvation of the vegetative cell and describes the transformation from the vegetative Bacillus to a non-growing very resistant endospore[22]. The transformation from the vegetative cell to the dormant endospore takes approximately 8 hours[28]. During the sporulation process the organism passes a complex series of events in cellular differentiation while forming an endospore, arranged in 8 stages. The process needs the synthesis of many proteins and the activation of endospore-specific genes in response to an environmental actuator to sporulate[26].

3.1 Initiation of sporulation

The initiation of sporulation is complex, but there are at least three types of input signals which are integrated by the cell before sporulation begins[29]:

1. Nutritional signal: Starvation for sources of carbon, nitrogen, or phosphorus can induce sporulation; good carbon sources, such as glucose, repress it.

2. Population density: sporulation could not be induced efficiently in cells maintained at a low population density – it seems that vegetative cells grown to a relatively high density produce a substance, possibly an oligopeptide that is necessary for efficient sporulation. How the cell could sense the rate of oligopeptide uptake, or interpret this information, is unknown.

3. Cell cycle: the sporulation cycle can occur only at a specific point in the cell division cycle.

3.2 Stage 0: the decision between vegetative growing and sporulation

The decision between further cell division and starting the sporulation process is essential for the future of the cell. The decision of remaining in a vegetative cell growing or initiating the sporulation process has to be made when either the exponential growth of cells is very high and therefore the density of the cells increases even more, so that nutrients are getting very bare, or simply the living conditions are getting extremely bad, so that survival is not ensured. If the organism has not enough resources for both daughter cells, which result from a normal

cell division and even not enough resources for the sporulation process, the vegetative cell will die. The decision of initiating the sporulation in time is very important for the cell to survive. This strategy is easy for Bacilli living in laboratory conditions. In nature the decision becomes much more difficult[26]. The organism has often to live with very spare nutrient resources, but it has developed many survival tricks, such as building antibiotics, which inhibits the further growing of other organisms. Additionally, a Bacillus can release enzymes, which help to extrapolate additional nutrients. If all these surviving strategies are not enough for surviving the vegetative cell decides to initiate the sporulation process. This decision is called commitment, and is irreversible till the sporulation process has finished. In addition to the external factors, which initiate the sporulation, there are a lot internal signals, which have a major function of committing the transformation to the dormant state. The sporulation program can be started at only one point during the cell cycle. In order that the sporulating program is efficient, the cell population has to exceed a certain amount of cell density depending on the nutrient sources. It is supposed, that the cells can communicate by excreting a certain substance, which gives information about the cell density of the whole culture. The internal process of initiating is very difficult. There are many genes which play a role in starting the whole process. The most important gene is the spo0 gene, which is responsible for the commitment decision and therefore the start of the sporulation. The second most important is the spo0A-gene. The more the amount of spo0A, the higher is the probability to start the sporulation program[28]. The communication process of the genes and the point of commitment are still not fully understood and have to be investigated more in the future.

3.3 Stage II and III: formation of the asymmetric division

The process of sporulation has now been initiated. About forty minutes after the commitment the cell division cycle passes the final round in replicating the DNA. Thirty minutes after the replication cycle the daughter cells segregate genophores. In contrast to the vegetative cells the division is asymmetric[23]. This is the first stadium of the sporulation process that is morphologically visible. Within the vegetative cell there are many molecular activities and decisions, which have to be made much earlier before a morphological differentiation of vegetative cells can be seen during the way of building endospores. Asymmetrical division is very characteristic for many differentiation processes. The exact process of the asymmetric

division is poorly understood, but there are a few models, which give an idea to understand the division process[28].

First there exists a mother cell containing two daughter chromosomes. At this point of time the cell is in an active growing phase and the next cycle of the replication phase of the daughter cells has started. Although the sporulation program has been initiated caused by the lack of nutrients, the replication cycle has to be finished, so that the mother cell consists in the end of two daughter pairs of chromosomes, and therefore four genophores[28].

The DNA replication of one of the two daughter chromosomes will be slower. If it is considered that the constriction of cell membrane happens after a completed DNA replication cycle, it seems to be clear that the side with the faster chromosomes will divide earlier than the side with the slower DNA replication. After finishing the last DNA replication cycle, a septum has to be created. The transcription of the genes of the mother and the daughter cells is very difficult to understand. It has to be said that one of the daughter chromosomes has to be in the future spore and the other three chromosomes will remain in the mother cell[26,28].

3.4 Stage IV and V: differential morphogenesis

The spore consists of the cortex and the coat, which builds outer part of the spore. The cortex is synthesized between the prespore membranes. At the same time the spore coat begins to be deposited on the outside surface of the spore. Within these two stages the mature spore becomes apparent. The mature spore has a distinct ovoid shape[29].

The final stages VI and VII are called maturation, with nearly no changes of the morphology. In this period the spore properties of resistance, dormancy and germinability are developed. In the end the mother cell lyses and the mature spore gets released[29].

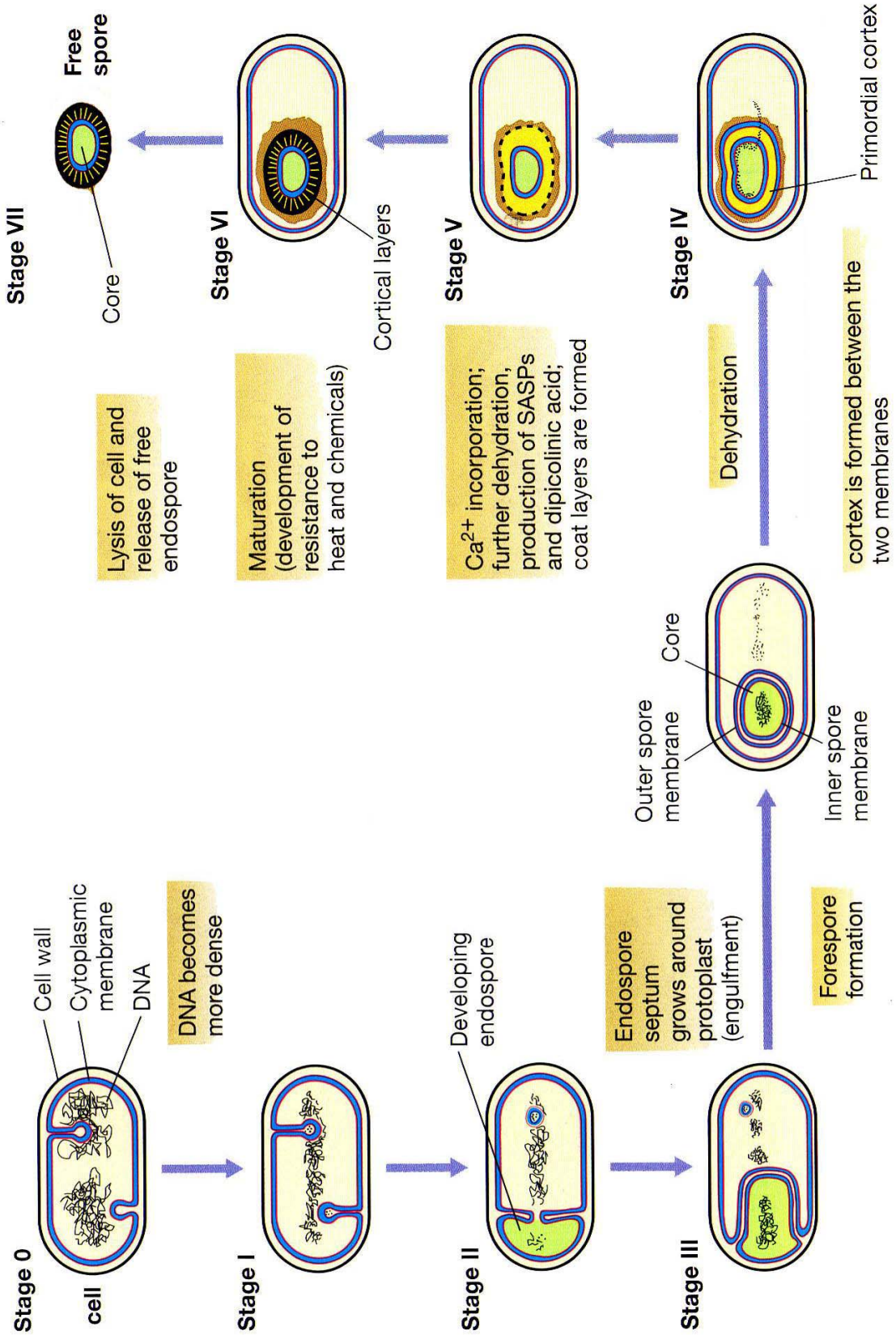


Figure 8: Stages of sporulation[26].

3.5 Genes

There are many genes controlling the sporulation process of bacilli. The sporulation program is characterised by the description of morphological and biochemical actions[28,29].

spo-genes:
The most important genes which affect the sporulation program, but not the vegetative cell division are the spo-genes. These genes act in a certain order of time, so that the following of each gene is depending on the progress of the gene before.

ger-genes:
They are responsible for the germination. There is no gene expression during the germination process and therefore the proteins for the germination have to be synthesized during forming the dormant spore and situated within the same.

cot-genes:
These genes are not responsible for regulating the sporulation program, but have their function in encoding the components of the structure of the spore coat.

3.6 Germination

Germination is the process of converting back from a dormant state to a vegetative cell. If the spore recognizes that the living conditions are appropriate again, the germination process will be activated. This process consists of three phases: the activation, the germination and the outgrowth.
In the laboratory, activation is triggered by heating the spores for several minutes at elevated temperature. After the activation, the spores have to be placed in a nutrient environment, commonly amino acids, such as alanine, which favours the germination process. If the activation process has not been induced by heating, the spores will remain in their dormant state, even when they are placed in a nutrient environment[26,30].
The germination process is characterized by the rupture of the spore coat. During this process the endospore swells and the metabolic activities will increase. There is an increase of Small Acid Soluble Proteins and a reduction of the calcium-dipicolinate complex. Therefore the cell looses its ability to resist their environment, i.e. heat, chemicals and radiation.

The final stage is the outgrowth, the new vegetative cell leaves the spore coat and is able to take up water, proteins, DNA and to perform the synthesis of RNA. Therefore the cell swells and remains in its vegetative state. The cell is now able to divided and produce more cells[26,30].

3.7 Resistance of *Bacillus subtilis* endospores

Bacillus subtilis endospores are known as the hardiest form of life on earth, which can resist extreme physical conditions. Due to the ability that bacilli can survive extreme terrestrial environments by building endospores, they can live many years. In their dormant state they can resist various physical challenges, such as heat, UV and gamma radiation, extreme desiccation, and oxidizing agents. During their inactivity the spores still monitor the nutritional status of their surroundings. If they recognize that the surrounding is appropriate again for living, they start the reversible process of sporulation, the germination. Capability of germination into vegetative cells is the indicator that endospores can survive in a dormant state for a long period of time. In 1995 scientists recovered spores from the gut of a bee, which was trapped in Dominican amber of a known geological age 25-40 million years ago. These bacteria, living in the bees´ digestive tract had turned into spores a long time ago and after placing them in a suitable culture, they turned back to vegetative bacteria[31]. The bacilli have to be prepared to survive against all possibilities of starvation. They must prevent damage which would inactivate critical cellular components needed for successful resumption of growth or repair or even replace those damaged critical components during germination, before their inactivation results in cell death[32]. In laboratory models many physical extremes have been tested. It has been investigated at which conditions the spores can survive and which biological mechanisms are responsible for these outstanding survival techniques. Studies have shown that the modulation of the sporulation condition has a significant influence on the resistance of the spore. These modulations include metal ion concentration, temperature and nutrients addition or deficiency. The main attention according to the question of surviving ability has been paid on spore coat resistance, core permeability, core water content, spore mineral content, repair mechanisms of DNA by many microbiologists.

Spore coats

The spore coats have the function to protect the genetic information of the *Bacillus subtilis* from their destructible environment, especially from hazardous chemicals. Depending on the

water content during the sporulation process the coats are resistant against heat, which was tested in many laboratory models. The coat provides protection against UV-radiation which affects the DNA situated in the core[33].

Core permeability

The endospore coats have a relatively low permeability for particles which are bigger than 200 Da[2)]. The spore has two membranes, but it is still unknown which of the two membranes is the responsible restricting barrier, the one that inhibits particles to enter the spore core[34].

Sporulation conditions

The resistance to certain environmental impacts such as heat resistance can be influenced by the sporulation conditions. It is known that by a variation of the ion concentration of the sporulation medium and at higher temperatures the core water content decreases and therefore the heat resistance increases. The sporulation conditions also have influence on other resistance mechanisms, such as UV resistance, but the survival mechanism is yet unknown[35].

Core water content

The content of water of the vegetative cells is higher (see chapter 2.4) than in endospores. Because of its influence on the heat resistance of the endospores, the core water content plays a major role during the sporulation process and is reduced during it[36].

Repair of DNA

Once the DNA of a cell is completely damaged the spore and the resulting cell will be dead. Therefore it is clear, that protecting the DNA from external endangering influences, such as UV-radiation, is indispensable. The DNA reparation mechanisms during germination and outgrowth are of supreme importance for surviving. Some enzymes in growing cells and some proteins in the dormant spore are responsible for inducing the DNA repair[32] mechanisms.

[2)] In molecular biology, the unit "Dalton" [Da] is often used. 1Da = 1u = 1.660538782(83) × 10^{-27} kg

4 AFM

The Atomic Force Microscope (AFM) was invented by Binning, Quate and Gerber in 1986[37]. It is a very high resolution type of scanning probe microscope for imaging, measuring and also manipulating matter. It offers the possibility to investigate the surface of conducting and non-conducting materials. In contrast to other high resolution microscopes, there are no mandatory requirements for vacuum or other preparation techniques, such as metallising[37]. AFM can be used in any environment, such as liquid by using a closed fluid cell, various gases, vacuum, at low temperatures (lower than 100K), which could be very useful to avoid thermal vibrations, as well at high temperatures[38]. Because of these outstanding properties, the AFM is a convenient tool for biological samples, without damaging them or changing their physical properties. The measuring principle is based on the fact that there are very small forces (<1μN) between the AFM tip and the sample surface. The prepared sample, usually fixed on a glass slide is mounted on a piezoelectric tube, which makes it possible to scan the sample in x- and y- direction. The main two possibilities of doing measurements with the AFM, are the contact mode (DC mode) and the dynamic mode (tapping, AC mode)[39]. The AFM can measure a variety of forces, down to tens of piconewtons, including van-der-Waals-forces, electrostatic forces, magnetic forces, adhesion forces and friction forces. There are also techniques for measuring electrical, mechanical and chemical properties of a sample[40].

4.1 Elements of AFM

The main parts of the AFM used in this work (MFP-3D Atomic Force Microscope^{TM}, Asylum research, Santa Barbara, CA, USA) are the cantilever with a sharp tip at its end, which is used to scan the sample surface, an IR-laser, which is aligned with the cantilever and a deflection sensor, which is a photodiode detecting the position of the cantilever, a tube scanner, which provides the movement in x- and y-direction, an inverted optical microscope and a computer.

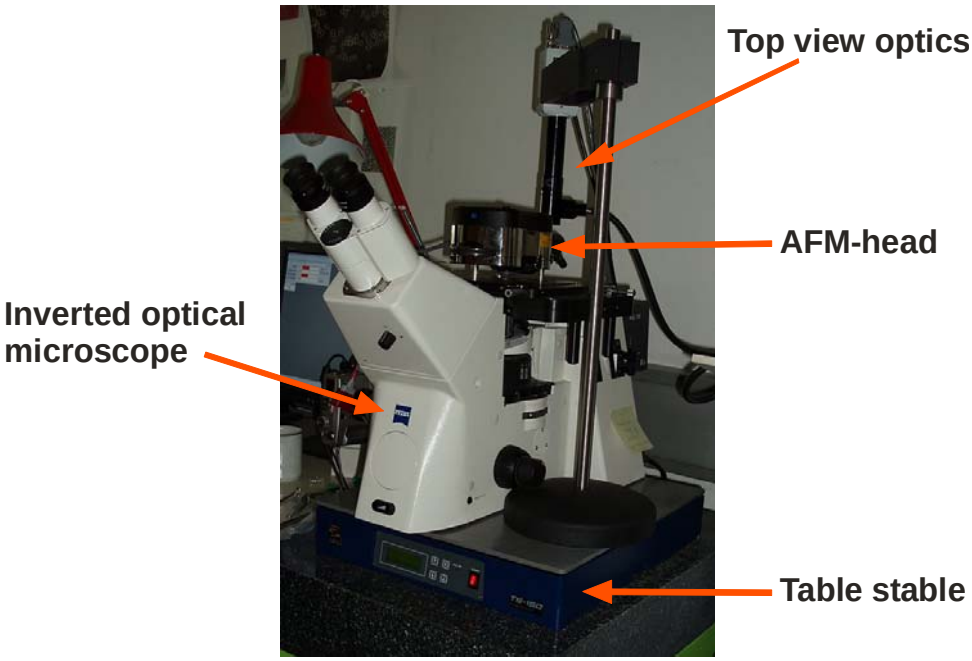


Figure 9: AFM and its components (MFP3D-Asyluum Research).

4.2 AFM Head

The AFM head comprises of the most sensitive parts of the AFM. Beside a lot of electronic devices, the main components of the AFM head are the IR-laser, the mirrors for adjusting the single components, the cantilever and the photosensitive detector.

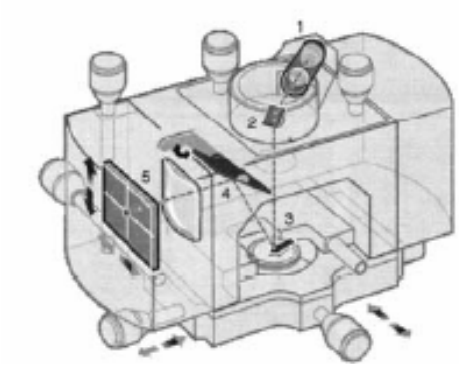


Figure 10: Inside of the AFM head. 1) Laser; 2) Mirror; 3) Cantilever; 4) Mirror; 5) Detector.

4.3 Cantilever

The quality and stability of cantilever tips have a major influence on the image quality. The ideal cantilever tip for topographical measurements has a very sharp tip, optimally one atom, without any lateral extension and is infinitely thin. Usually the tip is described by the tip radius and the beam width. Cantilevers operate at a certain resonance frequency, ranging from 5 to 300 kHz, depending on the geometry and the spring constant, available in a range from 0.01 to 100N/m[41]. The thickness of a cantilever is measured by interferometric microscope techniques, the length and width are measured with optical microscopes. If these parameters are known the resonance frequency and the force constants can be calculated considering the mass of the tip. Attractive and repulsive forces act on the cantilever tip, which are the reason why the cantilever gets bent, related to the spring constant. The correct setting parameters for imaging, such as scanning mode, scanning speed and the spring constant are almost all cantilever depending.

Common cantilevers are made from monocrystalline silicon or silicon nitride (Si_3N_4) by lithographic and etching techniques (see Figure 11). One method of producing cantilevers is the batch fabrication technique. By using this process an array of square openings is etched in a SiO_2 mask layer over a (100) silicon surface. By etching with KOH a pyramidal mold is created, which provides a mask for the cantilever. Then the etched mask is removed and another mask is applied to define the cantilever shapes with the pyramidal etch pits at the end. After filling the etch pit with Si_3N_4, the silicon is removed to free the cantilever and tips. This fabrication method provides a tip radius of less than 30nm[42].

Another method is to batch fabricate single-crystal Si cantilevers with integrated tips. It differs from the method described above in that way that a small mask is formed at the end of the cantilever. Then the Si around the mask is etched by KOH. This etching undercut results in a pyramidal silicon tip beneath the mask. Finally the mask is removed. Si tips are sharper than Si_3N_4 tips, because they are directly formed by the anisotropic etch in single crystal Si rather than using a etch pit mask for deposited material. This method provides a tip radius of less than 10nm[43].

Oxide sharpening improves tip sharpness and enhances tip asperities[43].

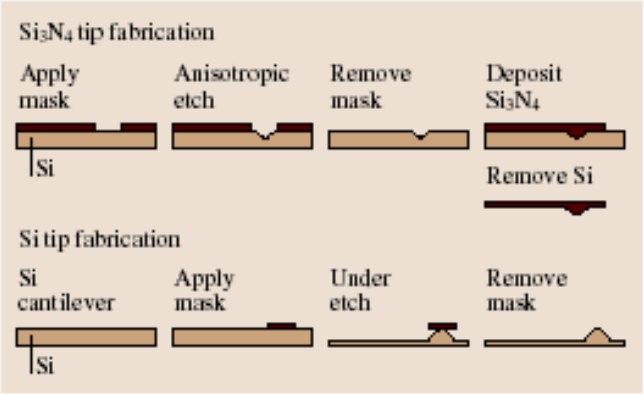


Figure 11: Tip fabrication technique[14]

The shape of the tip has an influence on the topography of a sample surface (see Figure 12). The obtained image is an interference of the shape of the tip and topography of the sample surface[44]. There can be falsifications of the image by recording artifacts of the tip. The shape and the chemical properties of the cantilever tip and therefore the interaction between the tip and the probe can also be affected by the adsorption of little particles of the tip. After recording a few images there can occur abrasions caused by hard sample surfaces as well as contaminations of the tip induced by organic particles.

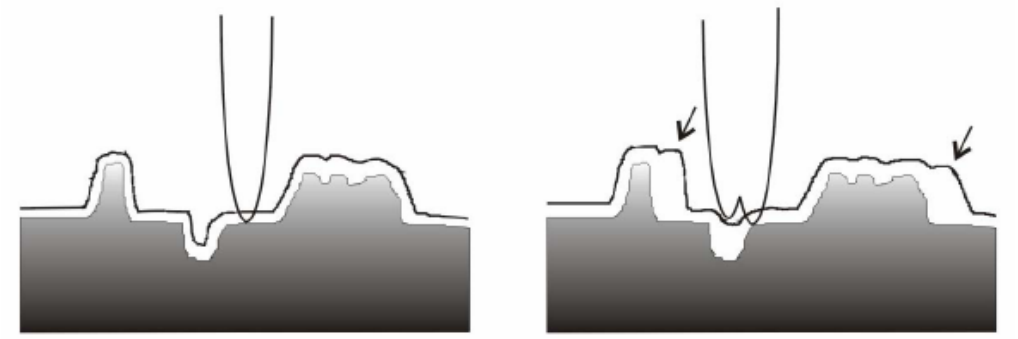
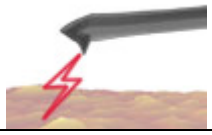
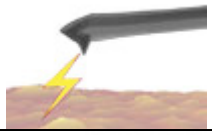
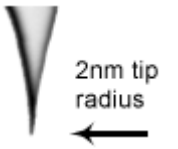
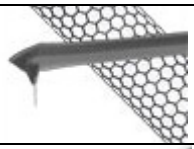
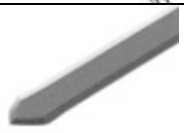


Figure 12: Tip shape influence on imaging the sample topography[44].

Depending on the measuring modes and sample properties there are many AFM probes available:

Modes	Description	AFM probe 1)
Dynamic, Non Contact	Dynamic mode AFM probes are used in air and fluid. They are useful for gentle tapping of soft and destructible surfaces.	
Contact	Contact AFM probes have low spring constants. They can be used in air and fluids and are usually used for hard surfaces.	
Force Modulation	The force constant of this type of AFM probe spans the gap between contact and non-contact mode and is specially tailored for imaging in force modulation mode	
Magnetic Force Mode (MFM)	MFM probes are made for magnetic force microscopy. They have a specific magnetic coating on their tip in order to feel magnetic forces.	
Electrostatic Force Mode(EFM)	EFM probes have electrically conductive metal coatings for electrostatic force measurements.	
Conducting Tip	AFM probes with coating on both sides of the sensor allow electrical contact between the tip and the sample.	
High Aspect Ratio	This AFM probe is for depth or step height measurements of high aspect ratio structures. They are fabricated with different tip lengths and shapes.	
Super Sharp	Super Sharp AFM probes are used for enhanced resolution imaging. They have a typical tip radius of only 2nm. They can be used for dynamic mode, force modulation mode, and contact mode.	
Carbon Nanotube Tip (CNT)	CNT probes have multi-wall nanotubes mounted at the end of their AFM tip and the best aspect ratio.	
Tipless	These levers are designed for special applications that do not require a tip at the end of the cantilever. They can be used for functionalizing or sensing applications or for attaching objects to the free end of the cantilever.	

1) Images taken from www.nanoscience.com

4.4 Force sensor

The microscope uses a flexible cantilever that bends and responses to forces between the tip and the sample surface. The more a specific cantilever is deflected, the larger the force. The deflection is today generally measured by an optical technique: a light beam, emitted by a diode laser, usually 5mW max peak output at 670nm (infrared)[45], forms a spot on the back of the cantilever which gets reflected to a screen. The screen itself is a position sensitive detector, which is divided into four quadrants and converts the spot position into an electrical signal[46].

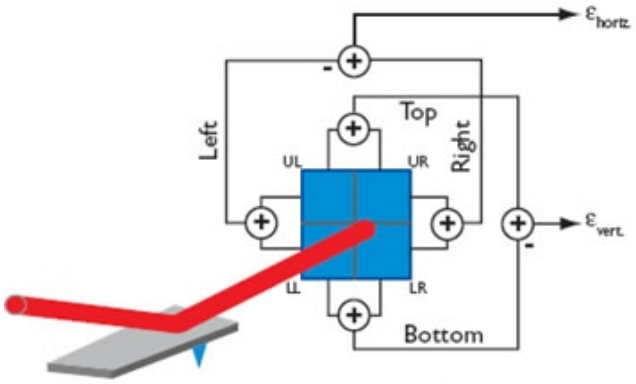


Figure 13: Diagram of a position sensitive detector.

4.5 Positioner

4.5.1 Tube scanner (one axis)

A tube scanner is responsible for positioning and moving the sample positioner in x- and y-direction and the scanner in z-direction. The positioners are made of piezoelectric materials, which have the property to change their crystal structure when a voltage is applied. This voltage application causes an expansion or a retraction of the crystal. The movement of a piezoelectric material can be accurately dispensed and is possible in very small ranges[42]. When a piezo shrinks in one direction the other two dimensions have to expand and vice versa. A positive voltage applied to the outside of a piezo tube affects the expansion radially and therefore the tube has to shrink in the z-direction. If the voltage is reversed the shell gets thinner and so the tube has to expand in z-direction. Since the tube is much longer as it is thick it ends up moving much more in z-direction than it does it radially[47].

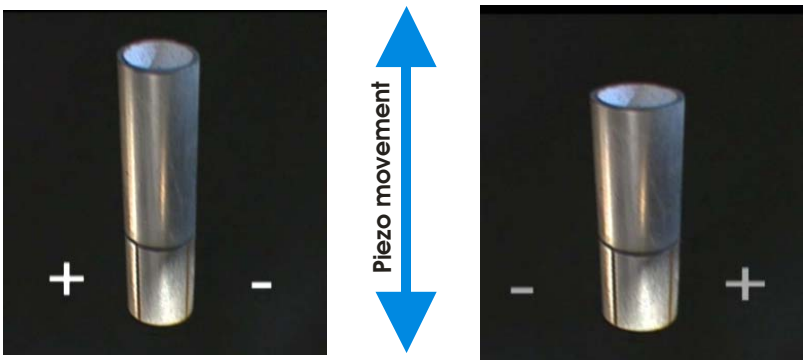


Figure 14: Characteristic of a single axis positioner[47].

4.5.2 Tube scanner (two axes)

The movement in x- and y-direction is more difficult. The tube is divided into four segments and therefore voltages can be applied to different sides of the tube[48].

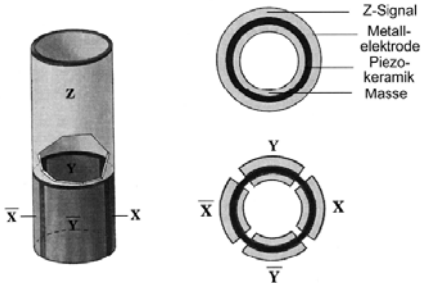


Figure 15: Schematic view of a four segment piezo tube[44].

If a positive voltage is applied to the outside of the tube, it expands and gets thicker, positive voltage on the inside of the tube causes shrinking of the tube. This adjustment creates a tube that tilts over.

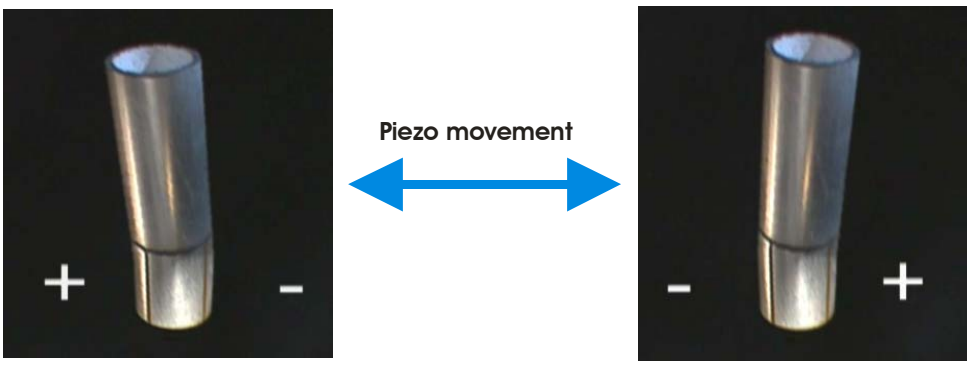


Figure 16: Movement of the piezo tube in x and y direction[47].

The main problem of using piezo tubes for nanoscale motion is coupling. If the piezo moves in x-direction there is also a little motion in y-direction and even in z-direction, so that the motion in x-direction is coupled to the motion in y- and z-direction. If coupling between different directions occurs there will be distortions in the images. Flexures solve the problem of coupling. Flexures have components which provide smooth motions, such as tiny springs (see Figure 17) along each direction without coupling. Because of the flexing instead of the sliding the friction is neglible and so tiny distances can be moved. The flexure scanner advantage over traditional piezoelectric tube scanners is that they have a minimal amount of bow in the z-axis.

In the case of the MFP-3D, the AFM offered by asylum Research, Santa Barbara, CA) the AFM sample table contains piezos, which are different from tubes, called stacks, which can move only in one dimension. A stack can expand about 45µm with an enourmous force. This is very important to push the tiny springs of the sample holder and therefore move the sample table[47].

If a voltage is applied on the piezo stack it expands and pushes the metal bar. The metal bars flexes and pull on the central stage. The central stage is held on the rest of the sample table by little springs and so the movement of the x-axis and the y-axis is completely independent[47].

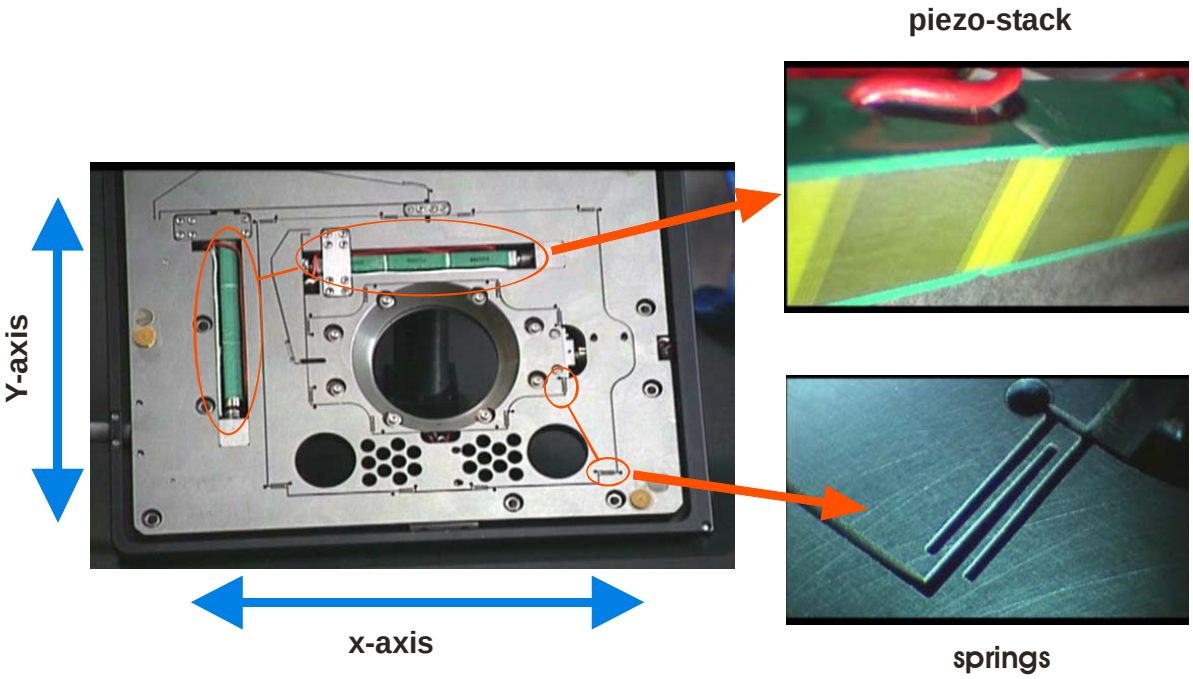


Figure 17: Bottom view of the central stage.

4.5.3 Linear Variable Differential Transformer

The position of the piezo is determined by using LVDTs (Linear Variable Differential Transformer). A LVDT is a sensor, which can detect changes of capacities and inductivities. With this information, drift effects and hysteresis effects are compensated. The Linear variable differential transformer is a type of electrical transformer used for measuring linear displacement. Current is driven through the primary coil at A, causing an induction current to be generated through the secondary coils at B. Displacing the core in one direction, the voltage in one coil increases as the other decreases, causing the output voltage to increase from zero to a maximum. The magnitude of the output is linear to the displacement of the core, the phase is indicating the direction of the displacement. An advantage of the LVDT is that the core is not touching the inside of the tube, so LVDTs are often used for position feedback. They are sensitive enough to digitalize the movements on a nanoscale[39].

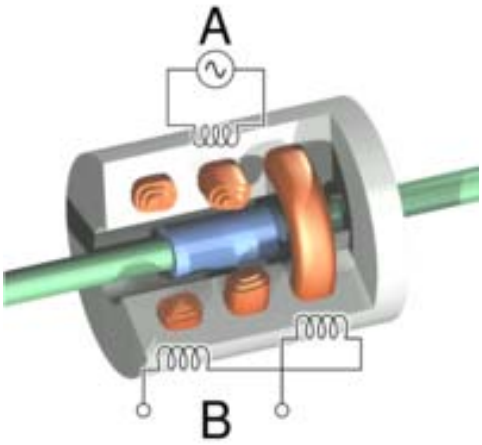


Figure 18: Cutaway view of a LVDT.

4.6 AFM Imaging Modes

4.6.1 Contact mode

In contact mode the cantilever tip is in close contact with the surface of the sample. During the first contact, the atoms of the cantilever tip sense a repulsive force, which is caused by the overlap of the electronic orbitals of the atoms of the sample surface. To scan the surface of the sample the tip has to be in contact with it. This causes a movement of the cantilever in z-direction and so changes of the deflection signal. The deflection signal is permanently sensed and compared in a DC feedback amplifier to a preset value of deflection, the set point. If the deflection value is different from the preset value, the feedback amplifier applies a voltage to the piezo to raise or lower the cantilever tip relative to the sample surface in order to restore the preset value of deflection. So the voltage to the piezo is directly correlated to the cantilever movement and the topography of the sample surface is given [49]. Because the tip is in hard contact with the surface, the stiffness of the lever needs to be less than the effective spring constant holding atoms together, which is on the order of 1 - 10 nN/nm. Most contact mode levers have a spring constant of < 1N/m. The advantage of using contact mode is that there is no introduction of any vibrations. Problems during measuring in contact mode can occur when the force applied to the cantilever is too high and therefore the tip scratches the sample surface. Additionally some samples, such as insulators and semiconductors trap electrostatic charges, which cause an additional force between the tip and the sample. These forces have an influence on the friction forces during the measurement, which are more destructive than the normal force applied by the cantilever during measuring in contact mode. To avoid the problems of destroying the sample surface one can use the dynamic mode[39].

4.6.2 Dynamic mode

In dynamic mode (tapping, AC mode) the distance between the tip of the cantilever and the sample surface is constant. This technique is used to avoid damaging the sample by scratching over it. In tapping mode the cantilever is sinusoidially vibrated by a piezo and oscillates at or close to its resonance frequency[49] in z-direction. The amplitude, the phase and the frequency are interacting by very small tip-sample interaction forces. These modifications provide information about the sample's characteristics. In contrast to contact mode, the cantilever oscillation amplitude is kept constant. The amplitude is permanently measured and a feed back loop adjusts the cantilever z-value due to the separation between the tip and the sample surface, which is defined by the setpoint-amplitude. Through this process the

topography of the sample surface is obtained. When the tip approaches the sample, the oscillation is damped and the reduced amplitude represents the feedback signal, rather than the DC deflection.

4.6.3 Phase Images in Dynamic Mode

The principle of phase imaging is based on using the dynamic mode measuring the phase shift of the oscillating cantilever relative to the driving signal. During the measurement there is not only a damping of the amplitude signal, but also a phase shift relative to the driving signal, which is caused by the interaction forces of the tip and the sample surface. The phase signal is recorded via a lock-in amplifier [50]. The resulting phase shift in air and also under water can be correlated with variations in material properties, such as friction, adhesion and viscoelasticity[51].

As mentioned above in chapter 4.2.6, in Dynamic Mode the cantilever is oscillating at or close to its resonance frequency. The amplitude, the phase and the frequency are interacting by tip-sample interaction forces. The obtained height and amplitude images are generally used for topographical AFM imaging. Phase imaging on the other hand is not only not appropriate to receive topographic contrast but it provides some additional information about the surface, more precisely the phase images can give us information about the viscoelastic properties of the sample surface. Thus it can be seen as an alternative method to force imaging without damaging the sample especially in the case of vegetative samples, like the *Bacillus subtilis*. To get an idea of the relation between the samples surface elasticity and the phase of the oscillating cantilever tip we use the damped driven harmonic oscillator approach [52],

$$m\frac{\partial^2 z}{\partial t^2} = -\alpha\frac{\partial z}{\partial t} - k\cdot z(t) - k\cdot z_0(t)$$

where the cantilever spring is supposed to obey Hooke's law with the spring constant k. The attenuation due to the viscoelastic behaviour of the tip-surface interaction is represented by the damping coefficient α, the driving oscillation is $z_0(t)$ and the tip position is $z(t)$ with

$$z_0(t)=A_0\cdot\cos(\omega t)$$

With some simple transformation we get [14]

$$z(t)+\frac{\alpha}{m}\cdot z(t)+\omega_0^2\cdot z(t)=A_0.\omega_0^2\cdot\cos(\omega t)$$

where ω_0 is the resonant frequency of the free undamped oscillator

$$\omega_0=\sqrt{\frac{k}{m}}$$

and Q is a quality factor, which describes the number of oscillation cycles, after which the damped oscillation amplitudes decays to $\frac{1}{e}$ of the initial amplitude.

$$Q=\frac{\omega_0\cdot m}{\alpha}$$

If we assume a measurement period long enough to reach a steady state, which is reached after 2Q oscillation cycles, for low scan rates the solution of the equation can be approached by:

$$z_s(t)=A_s\cdot\cos(\omega t+\varphi)$$

This transformation leads to the final expression for the phase φ[53], used for the phase images in Dynamic Mode AFM:

$$\varphi=\arctan\left(\frac{\alpha}{m}\cdot\frac{\omega}{\omega_0^2-\omega^2}\right)$$

This expression shows that the phase shift depends on the damping coefficient α and thus on the viscoelastic properties of the sample surface[47]. In this simplified approach of the damped driven harmonic oscillator we may consider two cases, a stiff surface and an elastic surface.

4.7 AFM resolution

The performance of the tips are characterized by imaging nanometer scale standards of known dimensions and the resolution is found to roughly correspond to the tip radius of curvature, the tip aspect ratio and the sample height. The topographical image of a feature is limited by the size of the probe tip, so the resolution is approximately the width of the tip. The resolution is of commercial ambient AFM tips is on the order of 5 to 10nm[44].

4.7.1 Resolution calculation

The principle of resolution of AFM is different from considerations of optical and radiation based microscopes. The resolution of optical and radiation based instruments depends on the wavelength of the radiation, while AFM is based on a three dimensional imaging technique. The ability to distinguish between the separation between two points limits the resolution of the AFM[54].

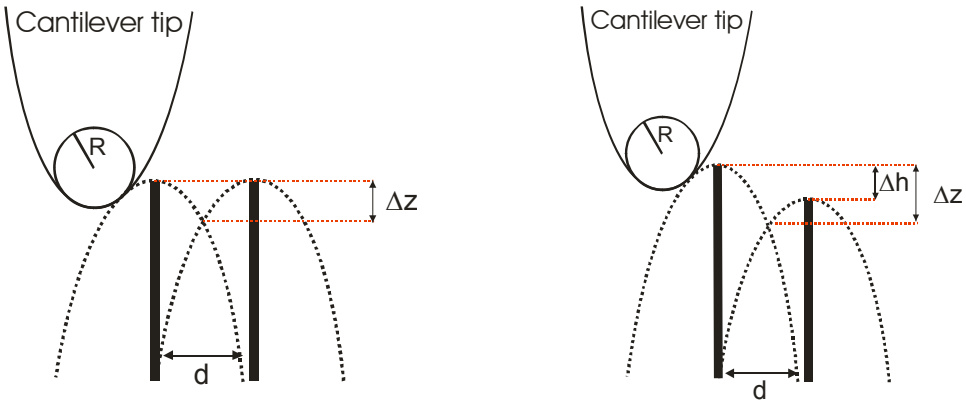


Figure 19: Resolution depending on the tip shape of a cantilever.

Considering two spikes within a sample surface (see Figure 19), there will be a small depression between the images of depth Δz. The spikes are resolved if Δz can be detected by the cantilever tip. The resolution d, which depends on the minimum separation of the peaks and the tip, is

$$d = 2 \cdot \sqrt{2R(\Delta z)}$$

If the peaks in the model have not equal heights (see Figure 19), the resolution will not be the same. If there is a height difference of Δh, the resolution is

$$d = \sqrt{2R} \cdot (\sqrt{\Delta z} + \sqrt{\Delta z + \Delta h})$$

4.8 Forces

The measuring principle of the AFM is the recording of interaction forces between the tip and the sample. Before executing a measurement, considerations about the acting forces and the

information one can get from them have to be performed. The forces are not only responsible for topographical information, but also for mechanical properties such as charges, elasticity and viscosity.

4.8.1 Cantilever

The deflection of the cantilever can be described by Hook´s law, which says that the force affecting the cantilever is proportional to its displacement from the idle state.

$$F_c = -k_c \cdot \Delta s_c$$

Where F_c is the force applied to the cantilever, k_c is the spring constant of the cantilever and s_c is the cantilever deflection.

4.8.2 Lennard-Jones-potential

The Lennard-Jones-potential describes the behaviour of attractive and repulsive forces between two atoms. The attractive forces are caused by the van-der-Waals forces, the repulsive forces are the result of the overlap of electron orbitals.

$$V(r) = \frac{a}{r^{12}} - \frac{b}{r^6}$$

where a and b are constants which are depending on the interaction of the two atoms. If there is a direct interaction between the tip and the sample surface, especially the Pauli-repulsion, resulting from the electron overlap will dominate, which is described in r^{12} within the Lennard-Jones-potential[55]. The van-der-Waals forces are explained with r^6 in the Lennard-Jones equation. The van-der-Waals force can be described as a result of interaction between permanent or induced dipoles.

4.8.3 Force curves

To record the acting forces of a sample surface the measurement of force-distance curves has to be done. With these curves it is possible to observe bindings between single molecules and perform elasticity measurements of single cells. The AFM tip is approached towards and

retraced from the surface, while the force between the tip and the surface of the sample is observed. The force-distance curves are a function of the z-value and the deflection signal (LVDT-signal, see Chapter 4.5.3) of the cantilever [39].

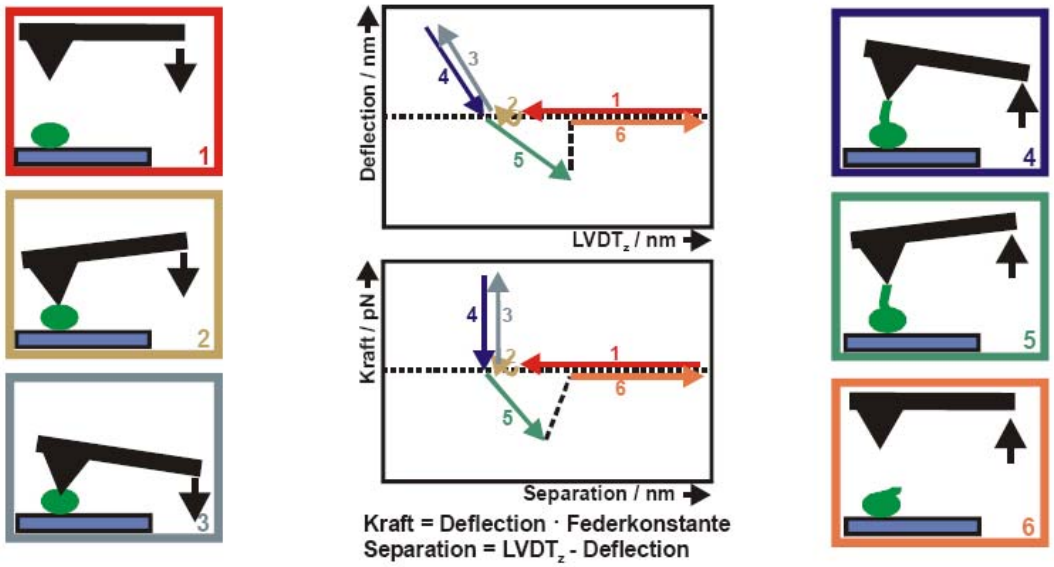


Figure 20: Force curves.

As seen in Figure 20 the position of the cantilever is far away from the sample surface and there is no movement of the cantilever (1), this means the deflection signal is zero. Then the AFM tip approaches the surface of the sample. Close to the contact of the tip and the sample surface, they are still not in contact (2), there is an attractive force between the atoms of the cantilever tip and the sample (Van-der-Waals force). Now the tip is in contact with the surface of the sample. If the z-voltage is increased, the cantilever starts to bend and there is an increase of the deflection signal (3). After decreasing the z-voltage of the cantilever the tip retracts from the surface and the value of the deflection signal is lower than zero, the adhesion area (4). Is the adhesion force lower than the pull off force of the cantilever, the tip disengages from the sample surface (5) and the cantilever moves back to the starting position (6). With this method it is possible to detect interactions between the tip and the sample[49] at piconewton force resolution.

The voltage-depending expansion of the z-piezo is monitored by a Linear variable differential transformer, a type of electrical transformer used for measuring linear displacement. The LVDT-signal measures the position of the head and not the position of the cantilever tip. Therefore the parameter separation is used. The separation is the difference between the LVDT signal and the deflection (see also chapter 4.5.3)[56].

5 Biological Applications of Atomic Force Microscopy

Bioimaging with the Atomic Force Microscope has become an ambitious field of research in the last two decades. It has increasingly been used to image microbiological samples at ultrahigh resolution. There are several advantages of using the AFM for biological investigations. Some of the applications are analysis of DNA, RNA, protein-nucleic acid complexes, chromosomes, cellular membranes proteins and peptides, molecular crystals, polymers and several biomaterials[57]. The AFM is a powerful tool for analyzing surfaces at ultrahigh resolution at ambient, fluid and vacuum conditions, providing three-dimensional images of structures with ultrahigh resolution. The AFM is not only an instrument for imaging sample surfaces, there is also the possibility to measure many physical properties such as mechanical properties, surface charges, molecular interactions, magnetic properties, friction forces and surface hydrophobicity[57]. The AFM techniques can also be used to manipulate living samples and surfaces.

The main advantage of using the AFM for biological specimens is the ability to analyze non-conducting surfaces without additional preparation like metalizing with gold which would have an influence on the biological properties of the samples. The second advantage is the non-destructive method of imaging the biological samples by using the dynamic mode of the AFM. There are scarcely other techniques, which can be used to obtain so many information of biological samples at such high resolution. By using the phase imaging mode (chapter 4.6.3.) of the AFM, surface components and their behavior can be recorded. So an idea of the inside of the sample could be given.

5.1 Substrates

Sample preparation of biological specimen has become a very important field in using the AFM for biological applications. In order to get reliable results it is very important to find a nondestructive preparation procedure, which provides that the sample is well attached to a solid substrate. Mica, glass and silicon nitride are appropriate substrates for fixing biological specimens. The fixation of the cells, organisms and biomolecules are of supreme importance for the sample preparation, because these biological structures have to be well attached to a solid substrate to resist the lateral forces which are exerted by the cantilever tip during the measurement.

There are a lot of substrates which are generally used for the investigation of biological samples with the ambient AFM[23,57]:

5.1.1 Mica

Mica is the most frequently used substrate for imaging biological samples. There are different types of mica and grades according to the ASTM grading scheme. The substrate has a layered structure and can easily be cleaved by using adhesive tapes to produce a clean, flat surface, which is negatively charged. The most commonly used mica substrate is the muscovite mica $KAI_2(OH)_2ALSi_3O_{10}$. The quality grade of mica is defined by the number of steps per unit area on freshly cleaved mica. Mica with a high grade of quality can also be used for AFM calibration studies.

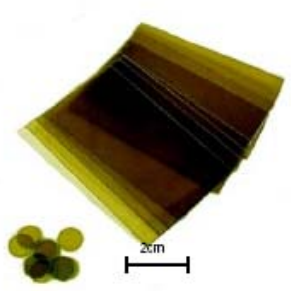


Figure 21: Samples of Mica structure for AFM measurements.

5.1.2 HOPG

A highly orientated pyrolytic graphite (HOPG) is a very common substrate for AFM measurements and can also be used for imaging biological samples. It is very flat over large areas and has hydrophobic properties, which can be very useful for immobilizing DNA. The plane structure of graphite makes HOPG a very anisotropic material with respect to electrical and thermal properties. The reason why this material is interesting for biological measurements is that it is very simple to create a very flat surface.

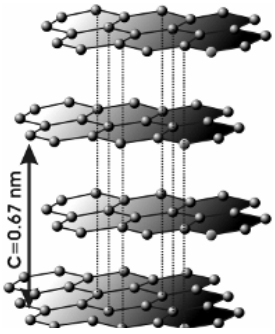


Figure 22: HOPG layers.

5.1.3 Glass slides

Glass slides are very easy to handle and flat enough for imaging cells and large biological structures. For imaging single bio-molecules the surface however is too rough. One problem is that glass slides are not adhesive and therefore the fixation of biological samples is not given. Another problem of using glass slides is the organic contamination of the substrate surface. These contaminations can be removed by washing the glass slide in concentrated acid solution followed by ultrasonication in water solution. For some biological measurements, like the analysis of immovable samples, glass slides can be a proper substrate.

5.1.4 Poly-L-lysine slides

Poly-L-lysine slides are glass slides coated with Poly-L-lysine. Poly-L-lysine is available in liquid form (see Appendix) and there are established protocols on how to coat glass slides. Poly-L-lysine is used in combination with other chemicals, i.e. glutaraldehyde as a cross-linker to improve the adhesiveness of the glass slides. By using such chemicals it has to be proven which influence they have on the mechanical and chemical properties of the biological sample.

5.1.5 Gel coated slides

Gel slides are common glass slides coated with a specific gel to improve the adhesiveness of dry biological samples, i.e. spore powder. Because these dry biological samples are not completely immobilized it can occur that the lateral forces of the AFM tip during the measurement can displace the sample, which leads to artifacts within the image. For measuring mechanical properties such as indentation forces, gel slides are not recommendable, because the gel does not represent a solid surface and will therefore falsify the results during the force mapping process.

Depending on the imaging methods and the data of interest different coatings can be used. A very useful coating is gold. Gold is chemically inert against oxygen and stable against radicals and can be easily modified with self-assembled monolayers[58]. Sometimes it is useful to use polymer membranes and macromolecular gels to immobilize large biological objects, such as cells.

5.2 Immobilization

In most cases before imaging biological samples using the AFM proper considerations about the preparation and the immobilization have to be made. Immobilization of biomolecules can be performed by several methods. A drop of an aqueous solution containing the biomolecules can be situated on the substrate and then evaporated or dried with nitrogen. Another approach is to use cross-linker which allows the adsorption of the biomolecules for a given period of time and is finally rinsed. A very important point is the covalent attachment of biomolecules. By using photoreactive crosslinkers[59] the immobilization of proteins can be achieved. Covalent binding of molecules on AFM probes is also used for recording interactions and certain forces between molecules. These methods can also be used for cells. For example, if the substrate is coated with adhesion proteins, the immobilization will be enhanced and therefore actin filament dynamics under the cell membrane of cells can be observed[60]. In contrast to human and animal cells, which spread over the substrate, the immobilization process for microbial cells is much more difficult, because simple adsorption processes will lead to a displacement of the cells by the cantilever tip. A possibility to immobilize the cells mechanically is to use an agar gel or a porous membrane. Under certain conditions cells can be kept alive, i.e. the vegetative *Bacillus subtilis*, in a nutrient solution. By changing the living conditions in a growth medium or inducing some biological processes by adding some chemicals, it is possible to investigate cell activities and dynamics with the AFM, ambient as well as using a fluid cell. Lipid films on a substrate are often used to mimic biological surfaces and therefore the properties of biological samples, e.g. enzymatic catalysis, cell adhesion, molecular recognition, membrane fusion[61,62] can be investigated. Lipid molecules are generally spread onto a solid surface, evaporated and are then compressed at a constant pressure. A lot of care has to be taken by producing lipid films on solid surfaces in order to avoid artifacts and formation defects of the deposited structure. Lipid layers are not stable in water and are only useful for ambient AFM investigations.

5.3 Cell surface investigation

One interesting topic for medicine, biology, industry and ecology is nanoscopic investigation of cell surfaces. Cell walls have properties, that can provide information on the interaction of pathogens with tissues and the accumulation on implants[63] in medicine, show advantages in biotechnology, such as cell immobilization in reactors and water safety treatment, e.g.

Bacillus subtilis in water hygiene, and have many applications in industry, such as biofouling. The reason, why cell surfaces play such a huge role is their ability to interact with the environment and to protect the cytoplasm from outer dangers. Cell surfaces act as molecular sieves and control interfacial interactions, such as cell adhesion and aggregation. In order to understand these functions, the structural and physical properties of the cell surface have to be investigated. There are several methods[64,65] for investigating these properties: x-ray photoelectron spectroscopy, infrared spectroscopy, electrophoretic mobility measurements, electron microscopy, and many other chemical and technical methods, which all have the disadvantage of destroying useful information during the preparation process, because of the required cell manipulation. By using the AFM it is possible to investigate the cell surface properties under physiological conditions and at ultrahigh resolution. Investigating cells with the AFM is very difficult and well deliberated pre-preparations have to be made. One has to consider the proper substrate for immobilizing the cells. Immobilization itself is a very delicate procedure, as mentored in chapter 5.2. Many microbiological cells have the disadvantage that they do not spread over the substrate and so the contact area between the cell and the substrate is very small. A proper method has to be found which provides the fixation of the cells and which does not influence the cell surface properties by any chemicals. One elegant method is to trap cells in porous membranes[66]. This method does not affect the cell properties, but has the disadvantage that this trapping does only work for spherical cells and not for rod-shaped specimens, such as *Bacillus subtilis*. One of the current investigation topics is the change of the cell surface structure under native conditions and to visualize the effects on external agents. By using the AFM the influence of chemicals, enzymes, solvents, ions and antibiotics shows significant changes of the properties of microbiological cells[67]. This advantage provides that cell growth, budding[3] processes and the change in cell surface morphology resulting from treatment with external agents can be investigated in situ. Molecular interactions play a big role in understanding medical and biological processes and are essential for the study of human health care. By using an AFM equipped with a closed fluid cell, the living conditions and nutrients can be changed during the measurement in order to observe changes of cell structures and their morphology in different environments. The sample fixation, using a fluid cell, is much more difficult, but in some cases, especially for vegetative samples, it is indispensable to use a fluid cell.

Another useful AFM application is the cell-probe technique, where the cells are directly attached to the AFM probe. The cell-probe method is used for recording force-distance curves

[3] The process of forming a new organism by the protrusion of another organism is called budding.

between the AFM cell coated probe and the substrate surface. The recording of arrays of force-curves in the x-,y-plane is called force-volume-imaging[56] mode. It can be used to record force-distance curves between the cell-coated tip and a solid substrate. Another field of usage of the AFM is the nanomedical investigation. AFM cantilever tips functionalized with biomolecules are used to investigate forces between ligands and receptors. For obtaining representative results of the chemical properties and intermolecular forces of a biological sample surface, it is important to know the chemical characteristics of the cantilever. There have been many probes developed with a well defined tip-chemistry. These probes are usually functionalized with self-assembled monolayers (SAMs), which are very sensitive to chemicals and have a high spatial resolution[32]. For the investigation of cells with the AFM it is necessary to work in parallel with an optical microscope to control the cantilever tip approach to distinct cellular features[68].

5.3.1 Cell surface elasticity

The cell behavior influenced by growth, gene expression and cell cycle progression is closely related to changes in their physical properties[69]. The measurement of the changes of elasticity will provide a better understanding of these processes.

5.3.2 Indentation

The elasticity of the cell can be measured by using indentation techniques [70]. By pressing the cantilever with a pre-defined force into the sample surface, force-distance curves are obtained to determine the compressibility of the cell wall. The comparison of force-distance curves (see chapter 4.8.3) can give information about elasticity differences in various samples.

5.3.3 Limitation of AFM indentation technique

In the force mapping technique (see chapter 6.3.1) the AFM cantilever tip intends into the surface of the material. If the cantilever spring constant is known and the AFM is correctly calibrated the indentation is recorded by the deflection of the cantilever as a function of the Z-position and converted into a force distance-curve[71]. The choice of a probe which is stiff enough to make an indentation of reasonable depth is very important.

The main problem of performing mechanical property measurements is the lateral motion of the tip during the indentation process, caused by the geometrical limitations. If a cantilever tip is intended into a material two kinds of tip motion will occur, the tip translation and the tip rotation. If there is a translation of a certain angle α there will be a vertical tip displacement of Δh. If the cantilever tip has the possibility of a laterally movement, the bending of the cantilever will result in a translation of the base of the tip and also a tip rotation of α. This occurring tip motions will result in a tip displacement of Δx[72].

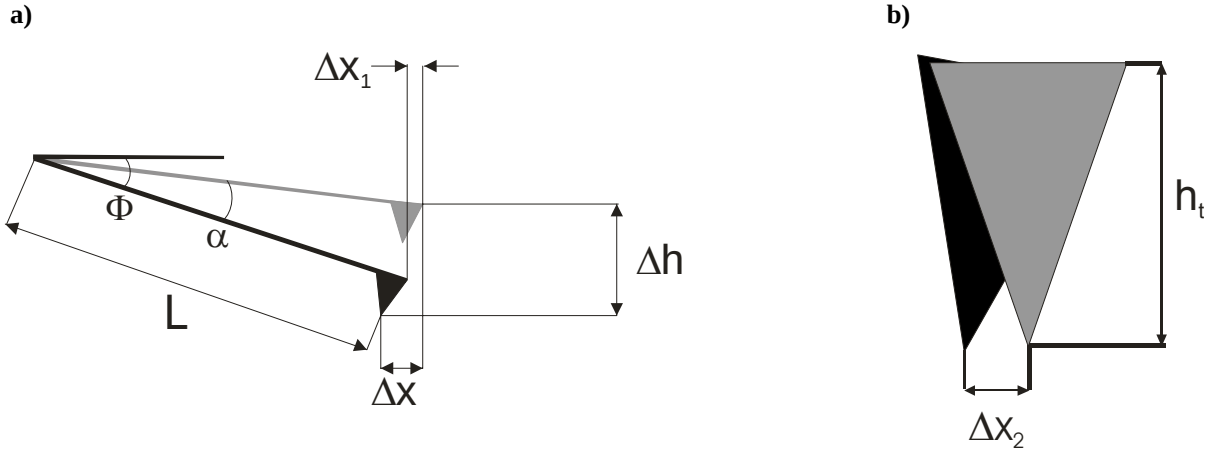


Figure 23: a) tip translation, b) tip rotation.

h_t.....tip height
L.....cantilever length
Θ.....cantilever mounting angle
α.....cantilever bend angle

Considering the geometry, a small bending will result in a tip displacement of

$$\Delta x = \Delta x_1 + \Delta x_2 = \Delta h \tan \Theta + h_t \alpha \cos \Theta$$

$$\alpha = \frac{3}{2} \frac{\Delta h}{L \cos \Theta} \quad [4])$$

$$\Delta x = \Delta h \{(\tan \Theta + \frac{3h_t}{2L})\}$$

[4] (see Appendix for derivation)

If the indentation displacement of the used 70kHz (Θ=11°; L=240µm; h_t=14µm; see Appendix) cantilever which were used for investigating the *Bacillus subtilis* is considered, there would be a displacement of the tip in the relation Δx=0,28Δh.

For solving the problem of displacement, there are several correction methods developed. It is not possible to fix a certain constant for each cantilever, which compensates the motion of the tip, because each material has a different influence on the behaviour of the displacement.

One method for correcting the displacement is the Deflection Lateral Correction (DLC)[72]. This method shows the required motion Δx to compensate the cantilever bend α. This compensation provides the mechanical measurements on heterogenous samples[75]. The implementation of the correction is given by multiplying a gain to the deflection signal and adding it to the piezo signal in x-direction. Usually the gain is determined experimentally by changing the setpoint in contact mode. Due to the change of the setpoint the deflection of the cantilever will also change. A correct gain is chosen when there is no lateral motion of tip in contact mode during changing the set point.

There can also be the possibility that the cantilever tip is constrained in motion, so that it cannot move the full displacement of Δx. If this happens the stiffness of the sample and therefore the restriction of the probe cause a bend in the cantilever end angle. This is caused by the lateral stiffness of the sample surface and the bending angle depends on the spring constant[71].

5.3.4 Cell phase imaging

By using the phase imaging mode several cell properties can be measured. As mentioned in chapter (5.3) it is possible to investigate the material properties, such as adhesion and elasticity by recording the phase shift as a function of the driving signal. Although this imaging method is not very easy to perform on living cells, the choice of the proper set point, the oscillation frequency and the scanning rate, this imaging method is evolving rapidly[73]. Forces, such as lateral and shear forces are reduced by using the dynamic mode, without damaging the cells. By using phase imaging the obtained image can be explained as a map of viscoelastic variations on the sample surface, and provide an insight of the cells. A positive phase shift means stiffer, a negative phase shift softer regions, and might be displayed by brighter and darker regions within the recorded image, depending on the contrast settings of the AFM software[74]. One influence of the phase shift signal is the surface stiffness, the second is the result of the viscoelasticity.

6 Material and Methods

The vegetative *Bacillus subtilis* cells and the spores were provided by the "Clinical Institute of Hygiene and Medical Microbiology of the Medical University Vienna". With the kind help of Regina Sommer, (Professor for Water Hygiene and head of the department Water Hygiene), the *Bacillus subtilis* cells were cultured and the sporulation process was induced. The samples of vegetative *Bacillus subtilis* cells in solution and the spores of *Bacillus subtilis* in solution as well as in dried form were investigated with AFM techniques. For the investigation the MFP-3D Atomic Force Microscope, (Asylum Research, Santa Barbara, CA, USA) in combination with IGOR Pro 5.05A, the controlling software, was used.
In the following an insight of the biological procedures of inducing the sporulation process, the preparation process of the samples, the morphological stages and the surface properties of UV-sensitive and UV-resistant endospores will be given.

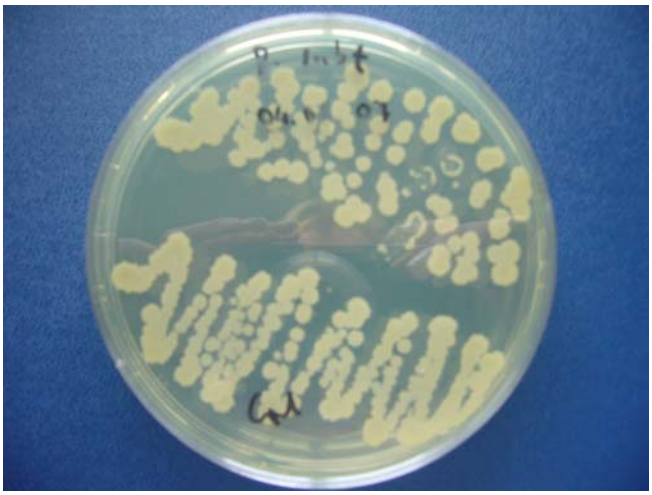


Figure 24*: Bacillus subtilis* culture, grown on Columbia agar.

6.1 Procedure for stimulating the sporulation process

There are two different types of *Bacillus subtilis* spores, the first type being relatively resistant to irradiation with a wavelength of 253,7nm (UV). The other type are spores which are less resistant to the same UV radiation and have been successfully established for water quality investigations especially for the biodosimetry of UV disinfection systems. The resistance to UV radiation depends on the method inducing the sporulation process and therefore which type of spore is formed.

6.1.1 UV-sensitive *Bacillus subtilis* spores

A procedure according to the Institute for Experimental Epidemiology (Wernigerode, Germany) was used for the spores production.

This method describes an accumulation technique on a solid culture medium with a pre-culture in bouillon[5]. For the production of spores Calciumchlorid-Columbia-Agar was used as medium.

Calciumchlorid-Columbia-Agar:

After preparing the agar medium and cooling it down to 50°C, a solution of 20ml/l 1% sterile calcium chloride is added to the agar. The calcium chloride raises the salt concentration, which finally stimulates the endospore formation.

Bacteria:

The *Bacillus subtilis* cells are taken from a 24 hour old culture on Columbia Agar.

Method:

1) Inoculating[6] of approximately 10 colonies each in 5ml TSB
2) Incubating in shaking bath at a temperature of 37°C for 4-5 hours
3) Transferring the pre-culture to the prepared $CaCl_2$- Agar –plates
4) Incubating the samples at a temperature of 37°C for 7 days
5) Harvesting the spores by washing the Agar-plates with sterile distilled water and centrifugation with 5000g for 15 minutes at a temperature of 5-10°C
6) Washing the bacteria three times with 20ml sterile distilled water and centrifugation them with 5000g for 15 minutes at a temperature of 5-10°C
7) Collecting the bacteria in 20ml culture medium
8) Heat treatment of the suspension in a shaking water bath for 10 minutes at a temperature of 80°C in order to inactivate the remaining vegetative bacteria

[5] Bouillon is a synonym for broth.
[6] Inoculating is a term used in chemistry and biology which describes the process of implanting microorganisms to a culture medium.

6.1.2 UV-resistant spores

A modified procedure according to Schaeffer [Munakata& Rupert] was developed for the spore production.

This method describes an accumulation technique by using a liquid medium (nutrient solution). For the production of spores the following culture medium was used:

Sporulation-Bouillon according to Schaeffer

Solution 1:

The first solution consists of a mixture of 280mg $MgSO_4 \cdot H_2O$, 1,11g KCl and 1ml $FeSO_4$ (31mg $FeSO_4 \cdot 7H_2O$ in 100ml distilled water) dissolved in 1l distilled water. After adjusting the pH to 7 it is autoclaved[7] with 8,9g Nutrient Broth No.2. (Fa. Oxoid CM 67),

Solution 2:

For the second solution 470mg $Ca(NO_3)_2$ and 390mg $MnCl_2 \cdot 4H_2O$ are suspended in 200ml distilled water and finally autoclaved.

For the final medium solution 1 and solution 2 are mixed 9:1.

The *Bacillus subtilis* cells are taken from a 24 hour old culture on Columbia Agar.

Method:

1) Transferring and inoculating 300ml sporulation medium in a 500ml Erlenmeyer flask
2) Incubating in shaking bath for 72 hours, at a temperature of 37°C
3) Treating the liquid culture in an ultrasonic bath for 2 minutes at a temperature of 10°C
4) Harvesting the spores by centrifugation with 5000g for 15 minutes at a temperature of 10°C
5) Washing the pellets three times with sterile distilled water and centrifugation with 5000g for 15 minutes at a temperature of 5-10°C
6) Collecting the bacteria in 200ml for culture medium
7) Heat treatment of the suspension in a shaking water bath for 10 minutes at a temperature of 80°C in order to inactivate the remaining vegetative bacteria
8) Treating the fluid culture in ultrasonic bath for 2 minutes at a temperature of 10°C

[7] Autoclaving is a method using pressurized devices to sterilize aqueous solutions by heating above their boiling point.

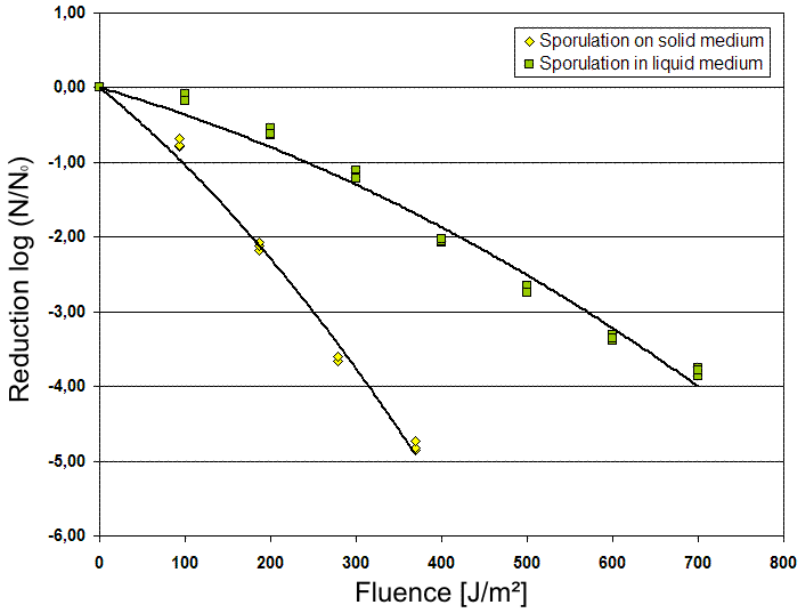


Figure 25: UV-253.7 nm inactivation of *Bacillus subtilis* spores, produced either on solid medium or in liquid medium[76].

6.2 Sample preparation

6.2.1 Substrate

In chapter 5.1 the importance of fixation due to forces induced by the cantilever tip and therefore the possibly of displacement of the cell or spore was discussed. To provide proper fixation of the sample, several substrates and chemicals were tested. The usage of glass slides in combination with poly-L-lysine and the usage of Polysine^{TM,8} slides proved to be the best substrates. The mechanical fixation of the vegetative *Bacillus subtilis* cells with porous membranes was out of the question, because filter membranes are not appropriate for rod-shaped bacteria (see chapter 5.3) and could induce artifacts on the elasticity measurements during the tip indentation.

6.2.2 Preparation of *Bacillus subtilis* spores in aqueous solution

The *Bacillus subtilis* spore solution is centrifuged for 15 minutes to separate the spores from agglomerates larger than the spores. About 50µl of the spore suspension is carefully pipetted

[8] Polysine^{TM} glass slides are made from a new adhesion technology which electrostatically and chemically attracts frozen as well as formalin, alcohol, Bouillon's or non cross-linking fixed tissue sections from animal, plant, and human sources. Polysine^{TM} slides are specially modified to attract and adhere both exfoliated and cultured cells. (adapted from Polysine^{TM}-cover)

and immobilized either on the Poly-L-Lysine coated glass slides or Polysine^{TM} slides. After fifteen minutes drying in air the samples are washed with PBS (phosphate buffered solution) in order to reduce the spore density (10^{10}/ml of the resistant and 10^{9}/ml of the non-resistant spores) of the spores on the substrate. According to the amount of PBS used by the washing procedure more or less *Bacillus subtilis* spores remain attached to the slide. The sample slides rest about thirty minutes in air before the first measurements are conducted.

Figure 26: Bacillus subtilis spores in aqueous solution.

6.2.3 Preparation of dry *Bacillus subtilis* spores

There are two preparation methods developed for immobilizing the spore powder to a substrate.

The first method is to put about 1ml Poly-L-lysine on a glass slide and smear it with a second slide. After 3 minutes waiting, the dry *Bacillus subtilis* spores are immobilized on the pretreated glass slide. After waiting for 5 minutes the loose spores are carefully tapped off and the fixed endospores remain on the coated glass slide. This preparation method has the advantage that there are nearly no artifacts, which are caused by the nutrient solution, but has the disadvantage that the fixation is not really good and setting the correct imaging parameters becomes very difficult.

The second preparation method is to dilute the dried spores with PBS 5 times. About 50µl of this solution is immobilized on with Poly-L-lysine pretreated glass slide or a Polysine^{TM} slide. After fifteen minutes drying in air the samples are washed with PBS (phosphate buffered solution) in order to remove the spores which are not fixed to the slide. The sample slides rest about thirty minutes in air before the first measurements are conducted.

6.2.4 Preparation of vegetative *Bacillus subtilis*

The vegetative *Bacillus subtilis* is usually dissolved in a nutrient solution. These nutrients can cause artifacts on the sample surface. To reduce the agglomerates a careful pretreatment has to be performed. First the vegetative samples are reduced by centrifuging the cell suspension for 15 minutes. This has been done at "Clinical Institute of Hygiene and Medical Microbiology-Water Hygiene of the Medical University Vienna". Due to the limited lifetime of the vegetative samples in a not appropriate environment and their ability to move on their own by the use of their flagella the immobilization is a very delicate procedure.

a) **b)**

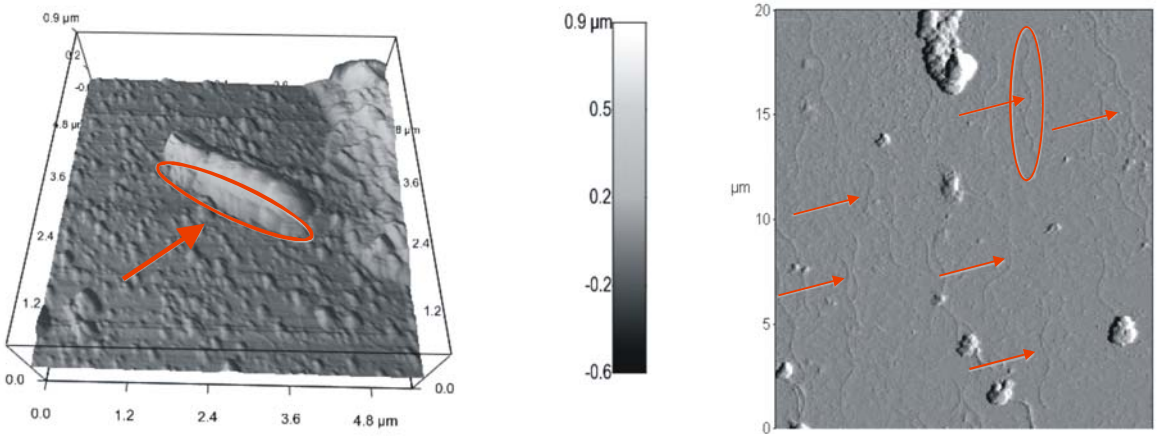


Figure 27: a) Particles of the nutrient solution attached to *Bacillus subtilis*; 3-D Heigth Trace 5.3µm x 5.3µm; b) Some flagella of Bacillus subtilis on PolysineTM slide, Amplitude Trace 20µm x 20 µm.

First, 50µl of the vegetative *Bacillus subtilis* solution is pipetted and carefully immobilized either on the poly-L-lysine coated glass slide or the Polysine^{TM} slides. Both substrates are ideally suited for the AFM measurements. Then the immobilized solution is smeared on the substrate by using a second slide. After 30 minutes drying in air the first measurements are conducted.

Figure 28: Vegetative Bacillus subtilis immobilized on a Polysine^{TM} slide.

It is very important to use PBS and not distilled water for diluting the spores and vegetative cells, because otherwise the cell membrane could burst due to the osmotic pressure.

6.3 AFM

For the measurements and the relevant information of the surface properties of the vegetative *Bacillis subtilis* and the spores, the MFP-3D Atomic Force Microscope™, Asylum research, Santa Barbara, CA, USA in combination with an optical inverted microscope is used. The resonance frequency of the cantilever used was approximately 70kHz, they had a spring constant of 1.2-3.0N/m (Olympus AC 240 TS, see Appendix). The calibration of the system is of enormous importance, because otherwise the obtained data have only arbitrary units.
First the signal of the laser beam, which detects the position of the cantilever, has to be optimized, i.e. by turning LDX[9] and LDY[8] (see Figure 29) until the sum meter is maximized. To minimize the deflection of the signal, the position sensitive detector has to be correctly adjusted by using the PD[8] wheel (see Figure 29).

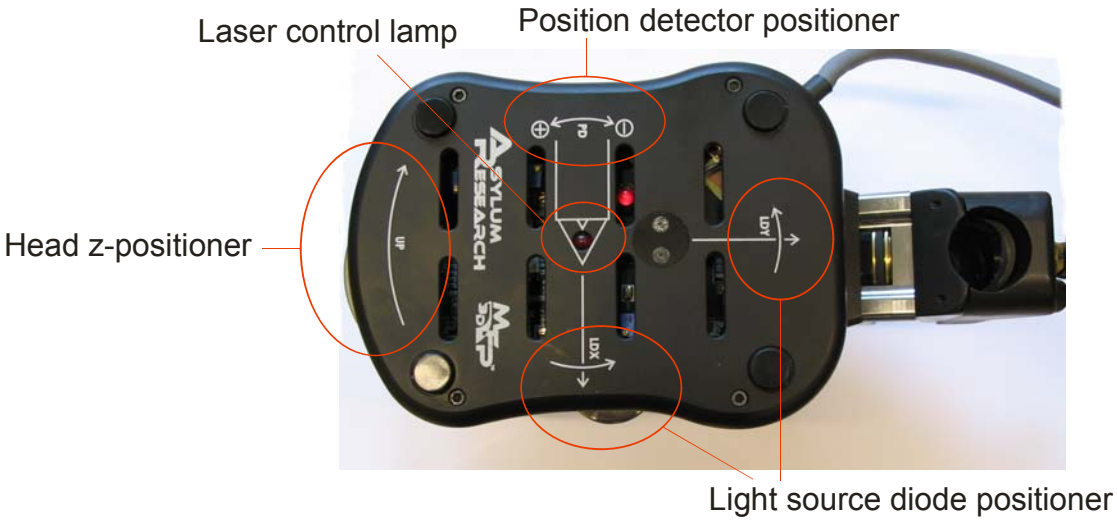


Figure 29: AFM head explanation.

The next step of calibrating the system is the setting of the correct resonance frequency. This can be done by measuring the Thermal Power Spectral Density, called Thermal Tune. The Thermal Tune measures the thermal noise of the cantilever and plots the results as the deflection vs. the frequency in kHz. A sharp peak within the graph refers to the resonance frequency of the cantilever and indicates that the system is working properly (see Figure 30.

[9] Especially for the MFP-3D Atomic Force Microscope™, Asylum research, Santa Barbara, CA, USA

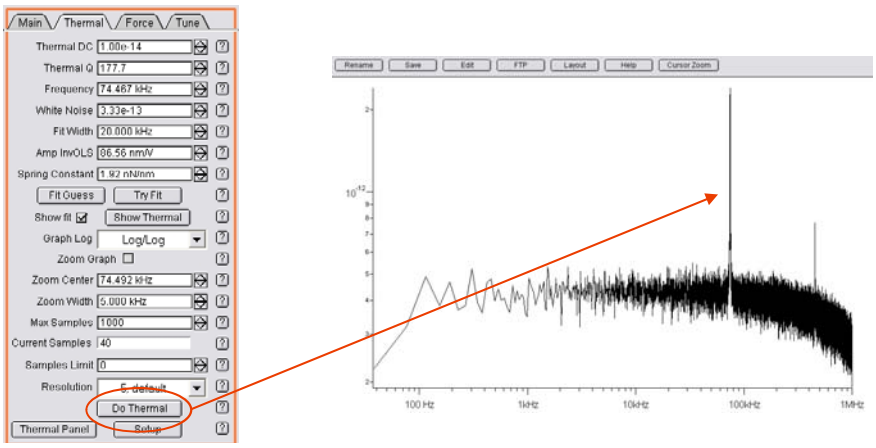


Figure 30: Thermal Tune, yielding the resonance frequency of the cantilever.

After verifying that the required alignments work, the system has to be tuned to the cantilever, by using the AutoTune function. This function dedicates the correct setting for the cantilever resonance frequency. The correct spring constant gets adjusted by calculating the slope the force-distance curve to one.

If everything is set properly and the automatically calculated values seem to be rational, the measurement can be done.

6.3.1 Force mapping

Because of the importance of working correctly with the IGOR Pro software using the force mapping mode, a detailed description of the working steps will be given. The Force Volume Panel (see Figure 31) by clicking *MFP Controls \ Force Mapping* has to be opened. The *Points per line* and *Number of lines* have to be set for the relevant measurement. By choosing *Number of lines* = 1, a mapping path can be created. One of the tracing windows have to be selected (Height, Amplitude, Phase) and the box beside *Show Array has* to be checked. Now green points (see Figure 33) get visible in the tracing window. After clicking *Edit Array* the points where the force curve will be acquired can be set manually.

a) **b)**

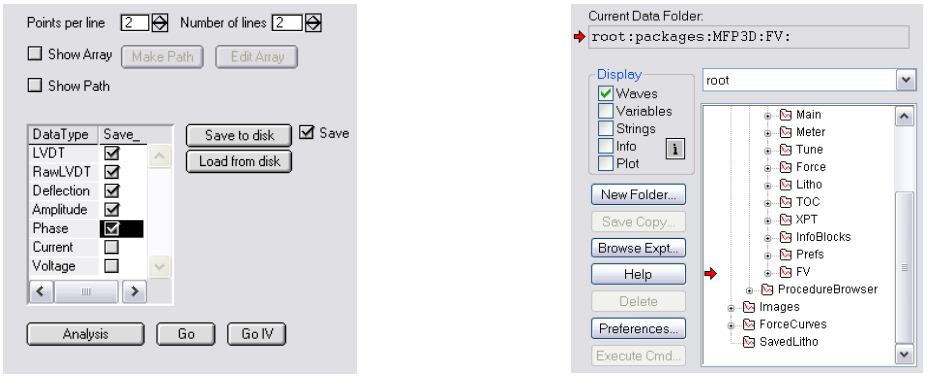


Figure 31: a) FVPanel; b) Data Browser.

The same arrangement of points can be used more than one time by saving the coordinates of the created path using the arrays of data for x and y: FVX and FVY. The Data Browser (*Data/Data Browser*) has to be opened and the red array within the Data Browser panel (standard is on root) has to be positioned on FV. Then the window *Data \ Save Waves \ Save Igor Binary* has to be opened (see Figure 32).

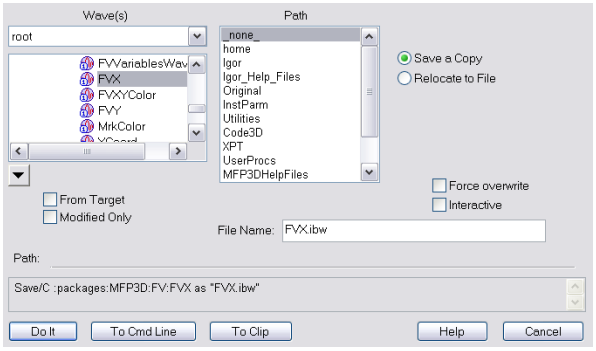


Figure 32: How to save Igor Binary.

Within the box “wave(s)” separately FVX and FVY have to be saved. To load the wave path, FVX then FVY within *Data \ Load Waves \ Load Igor Binary* has to be chosen and the saved arrangement of points will appear in the selected trace window. To manipulate the FVX or FVY wave e.g. FVX=some data (unit is meter) as a command in the command panel can be used. To manipulate only one single point it has to be addressed using [0] indices in brackets (Igor Pro starts counting indices with zero).

First the boxes beside the data which want to be saved should be checked. Using the Force Panel within the Master Panel the base suffix for the obtaining data can be defined. To start the measurements at the predefined points the *Go* button has to be pressed. For saving the curves it is important to check the box beside *Save*.

a) **b)**

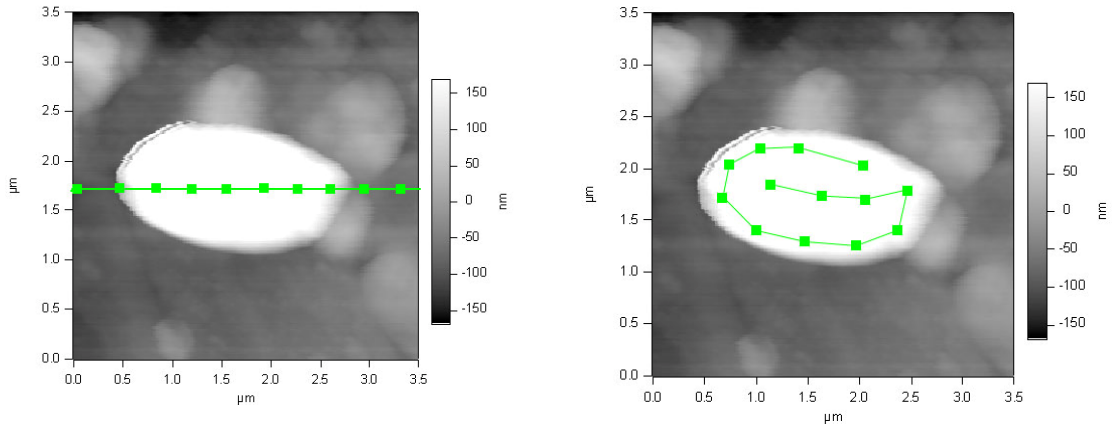


Figure 33: Force mapping of *Bacillus subtilis* spores: a) Force mapping points given on a line; b) Force mapping points arranged on a user-defined path.

For analyzing the saved data, the correct ensemble of force curves has to be chosen,

After loading the data, the relevant parameters have to be checked within the analyze panel. The run offline procedure is processing the data.

a) **b)**

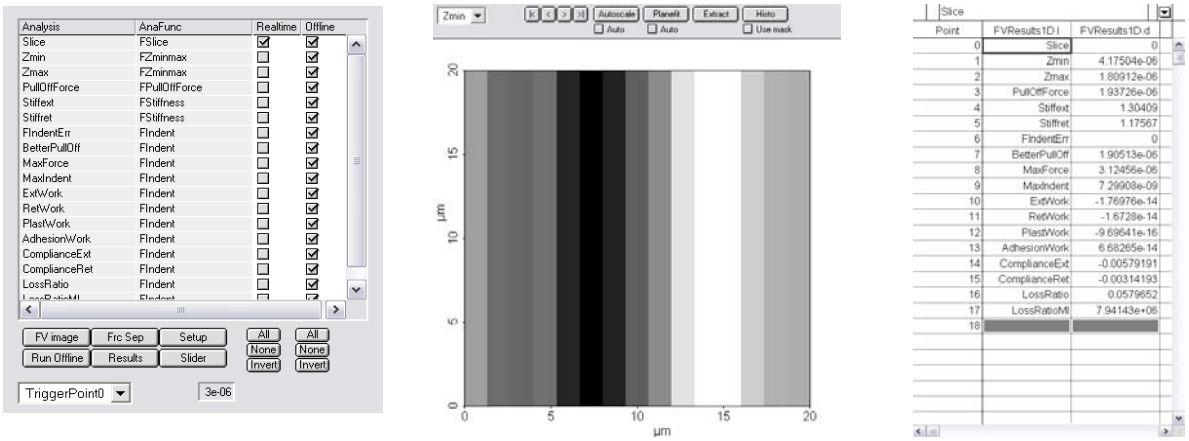


Figure 34: a) Analysis panel; b) FV Results.

In the FV Image the results appear in grey colours and each colour refers to one single force curve. Pressing *Results* will open a window with the data results.

For saving the analyzed data shown in the results table, a text file can be created, by setting the red array to FV in the Data Browser. Then *Save Delimited Text* in the Data folder has to be chosen.

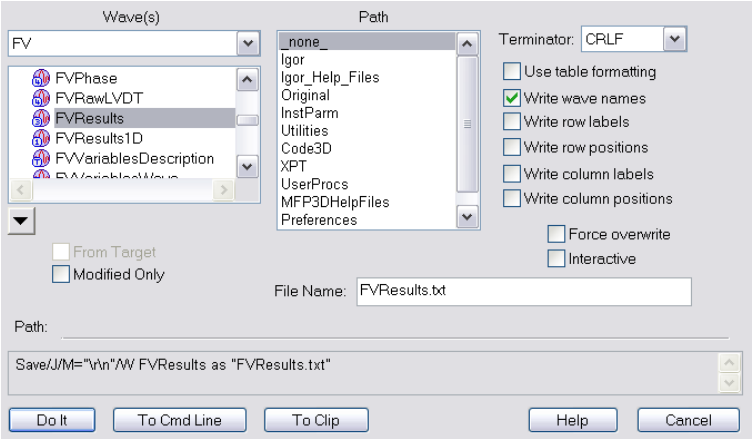


Figure 35: How to save the force mapping results.

6.3.2 Indentation calculation

The indentation depth of the cantilever tip into a sample due to a preset force trigger point is obtained by recording force-distance curves. The penetration depth is the parameter for the deformability of the cells. It is calculated by the position of the maximum movement of the piezo sensor in z-direction (z-max, according to the IGOR Pro-software), which is the coordinate when the sensor reaches the predefined trigger point, minus the position of the sensor when the tip is in first contact of the surface of the sample surface (a parameter called "Better Contact", in the IGOR Pro-software).

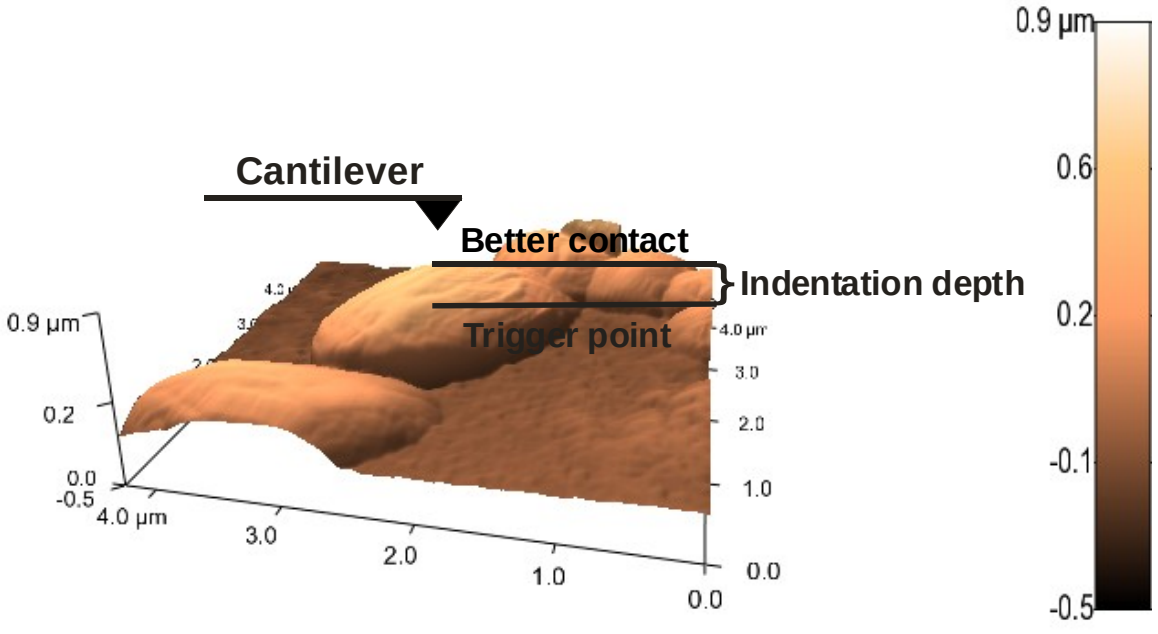


Figure 36: Indentation calculation; 3-D Height Trace; scan size 6.14µm x 6.14µm.

7 Results

The following images and results are obtained by using a 70kHz cantilever (Olympus AC240TS, f: 61.1-83.1kHz, k:1.2-3.0N/m). The preparation method depends on the sample, explained in chapter 6.2. The images are recorded using the dynamic mode. The scan frequencies and set points are sample depending. The images are investigated by scanning an area of 20µmx20µm and the regions of interest are finally magnified.

7.1 Imaging the sporulation process

The procedure of imaging the sporulation process is to induce the sporulation process of a culture of vegetative *Bacillus subtilis* and measure each day the progress of the sporulation. The sporulation process itself requires approximately 8 hours for one Bacillus, but approximately 7 days for the entire culture. There is the highest probability to image a vegetative cell on the very first day the sporulation has been induced. Between the first and the seventh day the probability to image a *Bacillus subtilis* in one of the specific stages of sporulation (see chapter 3) is very likely. Here for the very first time the sporulation process is imaged using the AFM.

Stage 0:

On the first day after inducing the sporulation process, mainly vegetative *Bacillus subtilis* are found within the cell culture. The sporulation program has not been initiated. The living conditions are still suitable for the vegetative *Bacillus subtilis*.

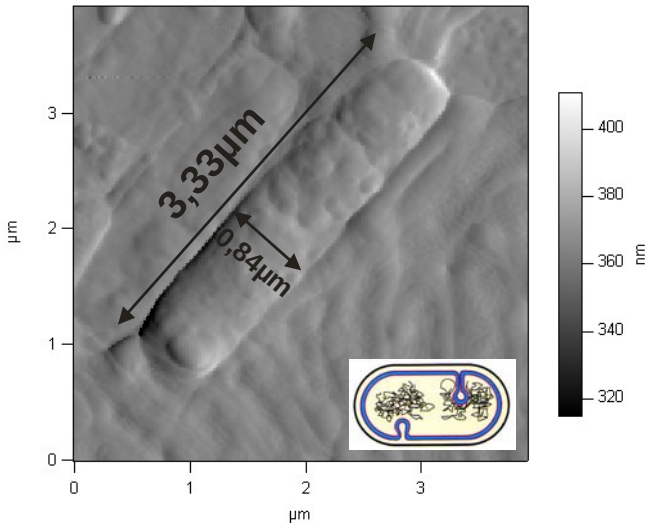


Figure 37: Vegetative *Bacillus subtilis* cell found in a cell culture on the day the sporulation has been induced; Amplitude trace; scan size 3.91µm x 3.91µm.

Stage I:

On the second day of sporulating, most vegetative cell give their commitment and decide to sporulate in order to survive the life threatening environment. The cell has to wait till a certain cell cycle point is reached, which allows the bacillus to start the sporulation program.

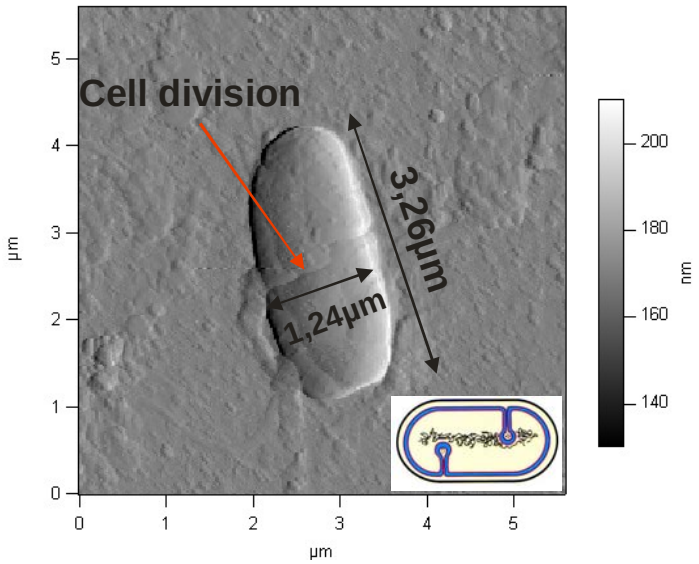


Figure 38: Vegetative *Bacillus subtilis* cell commiting the sporulation program; Amplitude Trace; scan size 5.59µm x 5.59µm.

Stage II:

On the third imaging day the process of nearly all cells for building an endospore is in full progress and generally cells within stage II of sporulating are found. In contrary to the vegetative cell the division is asymmetric and a morphological differentiation to vegetative cells can be seen. The cell has started to develop an endospore septum growing around the protoplasm.

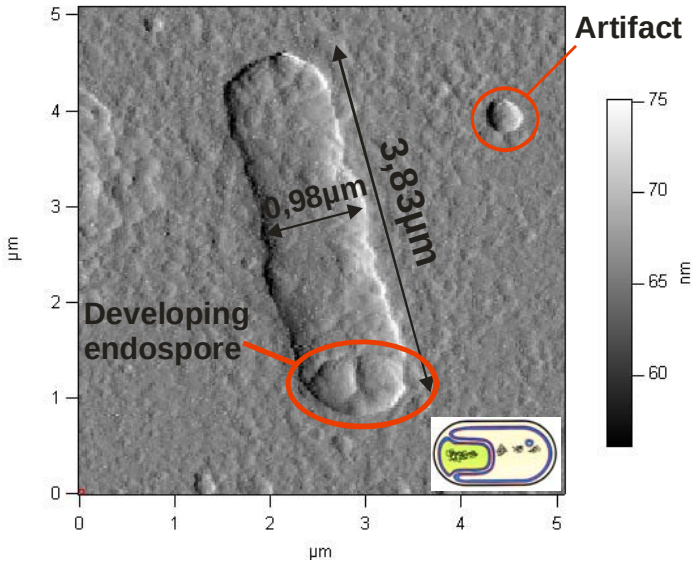


Figure 39: Process of building an endospore during the sporulation of the *Bacillus subtilis*; Amplitude Trace; scan size 5.07µm x 5.07µm.

Stage III, IV, V:

The forespore formation has nearly finished and the core is surrounded by an inner and an outer spore membrane. The dehydration starts and the primordial cortex is formed between the two membranes. The production of SASPs and dipicolinic acid has begun.

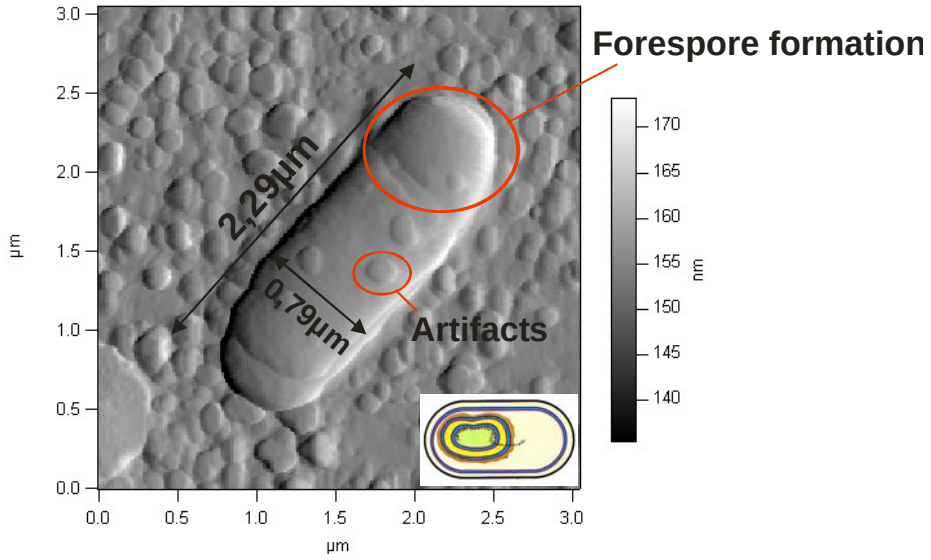


Figure 40: Forespore formation and core development of the *Bacillus subtilis* cell; Artifacts caused by the nutrient solution are attached to the cell surface; Amplitude Trace; scan size 3.04µm x 3.04µm.

Stage VI and VII:

On day 6 and 7 there are mainly spores found in the *Bacillus subtilis* samples. Occasionally some vegetative cells are imaged, which obviously had not started the sporulation program and died within the cell culture. Stage VI and VII are the stadium of maturation and the spore is resistant to heat, chemicals and radiation. The mature endospore releases from the mother cell, which finally lyses.

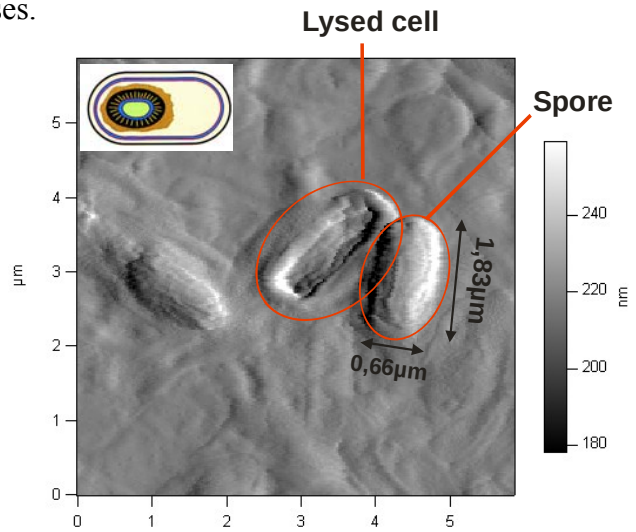


Figure 41: The spore releases the mother cell, which finally lyses; Amplitude Trace; scan size 5.86µm x 5.86µm.

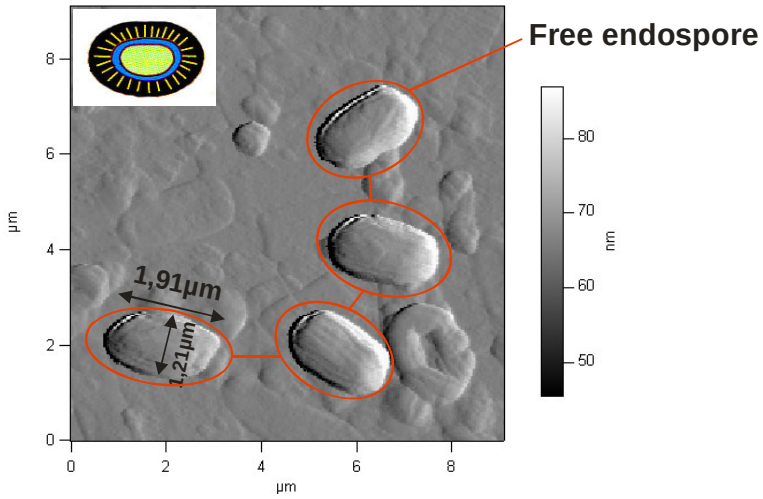


Figure 42: Almost only spores are seen within the cell culture 7 days after initiating the sporulation process: Amplitude Trace; scan size 9.08µm x 9.08µm.

Summary of the sporulation process:

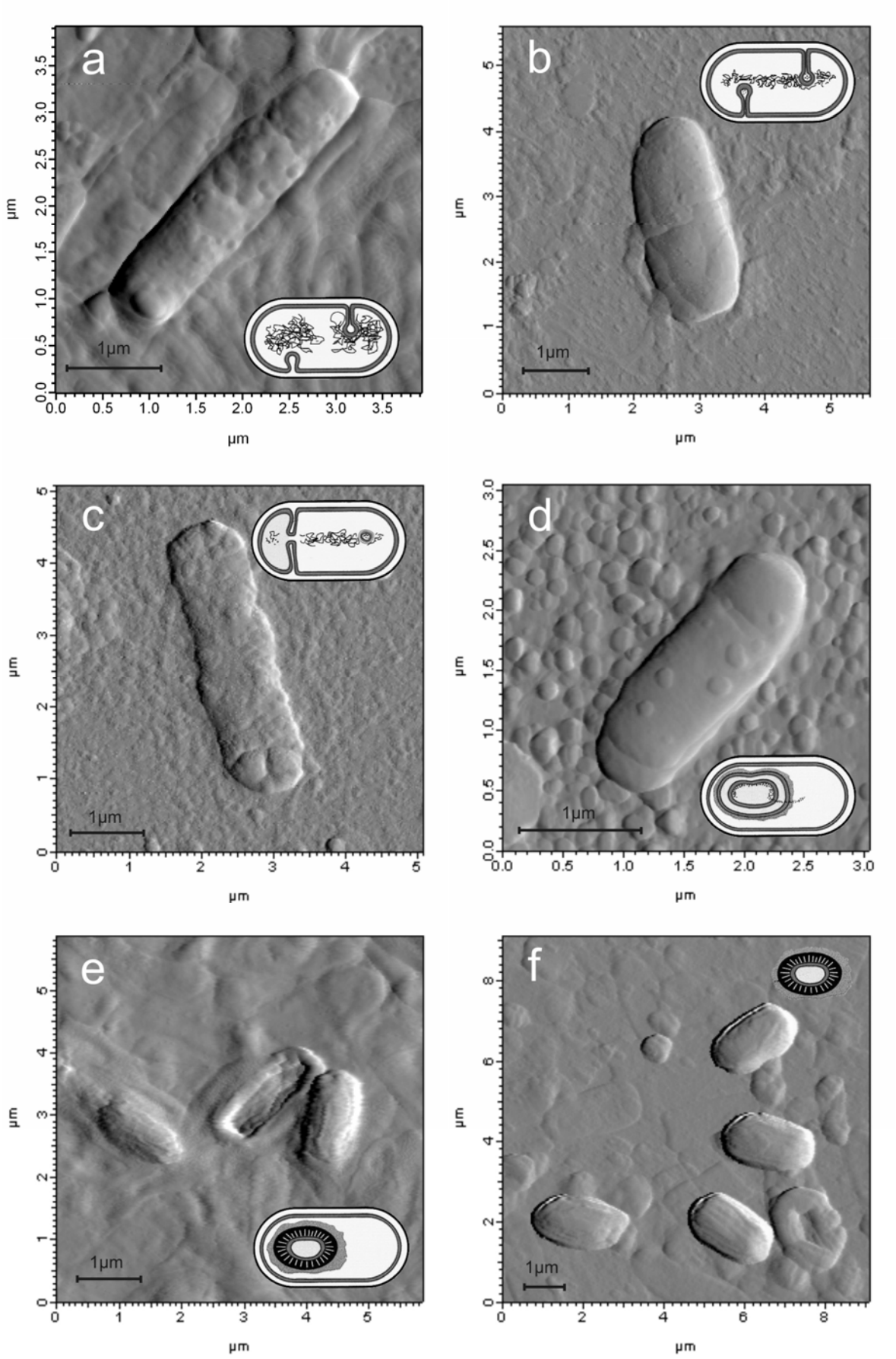


Figure 43: Summary of the sporulation process.

7.2 Imaging the *Bacillus subtilis* spores

Dependent on the method of inducing the sporulation of the vegetative *Bacillus subtilis*, there are differences in the UV-resistance of the endospores. A spore is considered non-UV-resistant, if the DNA gets damaged by the irradiation and the germination process cannot be started. There have been many investigations aimed at understanding the reasons for the differences of the UV-resistance, but there is still no explanation for this behaviour. By using the AFM phase imaging mode, new insights of the structure and elasticity of the spore are given. The following results provide new information about the different surface properties and hence consequences for the UV-resistance. Additionally, a force mapping path on each imaged spore is created and the indentation depth due to a preset indentation force at different indentation speeds is investigated.

The samples of seven different UV sensitive and seven UV-resistant endospore cultures, obtained from the "Clinical Institute of Hygiene and Medical Microbiology-Water Hygiene of the Medical University Vienna" in aqueous suspension are imaged using the Dynamic Mode of the AFM. The sample immobilisation follows the preparation method of the *Bacillus subtilis* spores in liquid described in chapter 6.2.2. First the prepared samples are scanned at low magnification using a scan size of 20x20µm^2 and the regions of interest are finally magnified (see Figure 44 as a typical example).

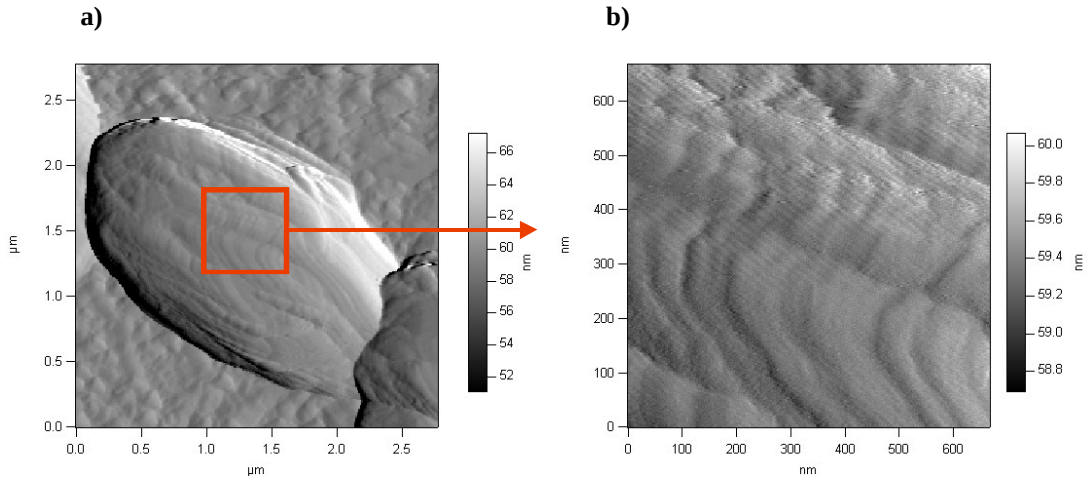


Figure 44: a) Typical Amplitude Trace of a UV-sensitive endospore; Scan size 2.7µm x 2.7µm, b) Amplitude Trace of the magnified region of interest of a UV-sensitive endospore; scan size 6.6µm x 6.6µm.

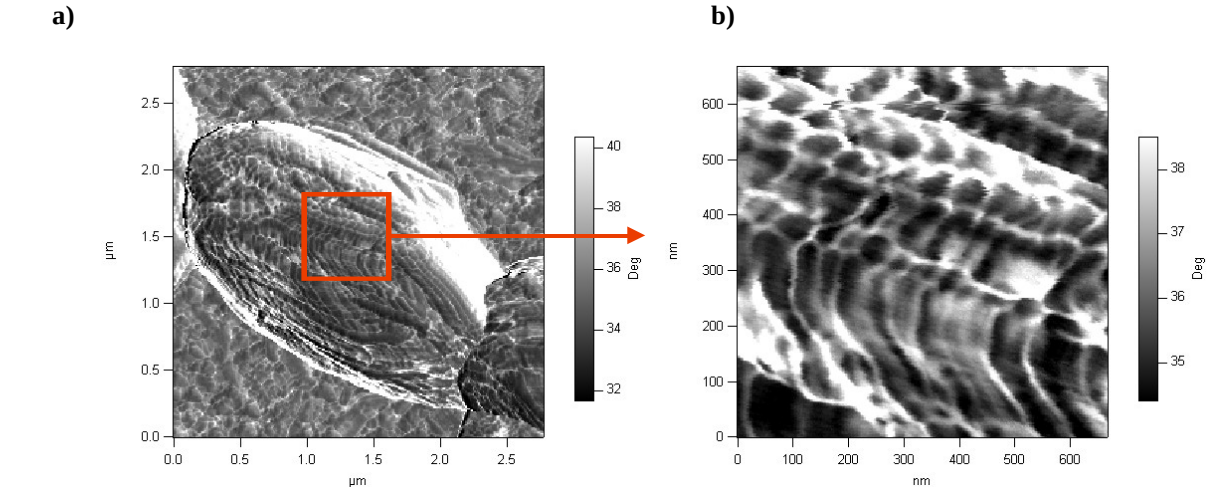


Figure 45: a) Typical Phase Trace of a UV-sensitive endospore; Scan size 2.7µm x 2.7µm, b) Phase Trace of the magnified region of interest of a UV-sensitive endospore; scan size 6.6µm x 6.6µm.

Considering the Phase Trace (see Figure 45) of the surface of the different spores, a difference in the structure compound which refers to different elasticity can be seen. The darker regions are softer and the brighter regions are stiffer. These mechanical differences in the stiffness of different regions of the spores´ compound possibly affect the surviving mechanisms against UV-radiation. The structure of the UV-sensitive spore is divided into small softer regions surrounded by a stiffer grid (see Figure 46). The UV-resistant spore has large regions that are stiffer than the rest of the spore. The different magnitudes of the softer and stiffer regions of the UV-sensitive and UV-resistant spores could have an influence on the UV-radiation absorption and therefore the protective mechanisms for the DNA. In Figure 46 the typical differences of elasticity of UV-resistant and UV-sensitive spores is shown, which is found in all of the recorded samples.

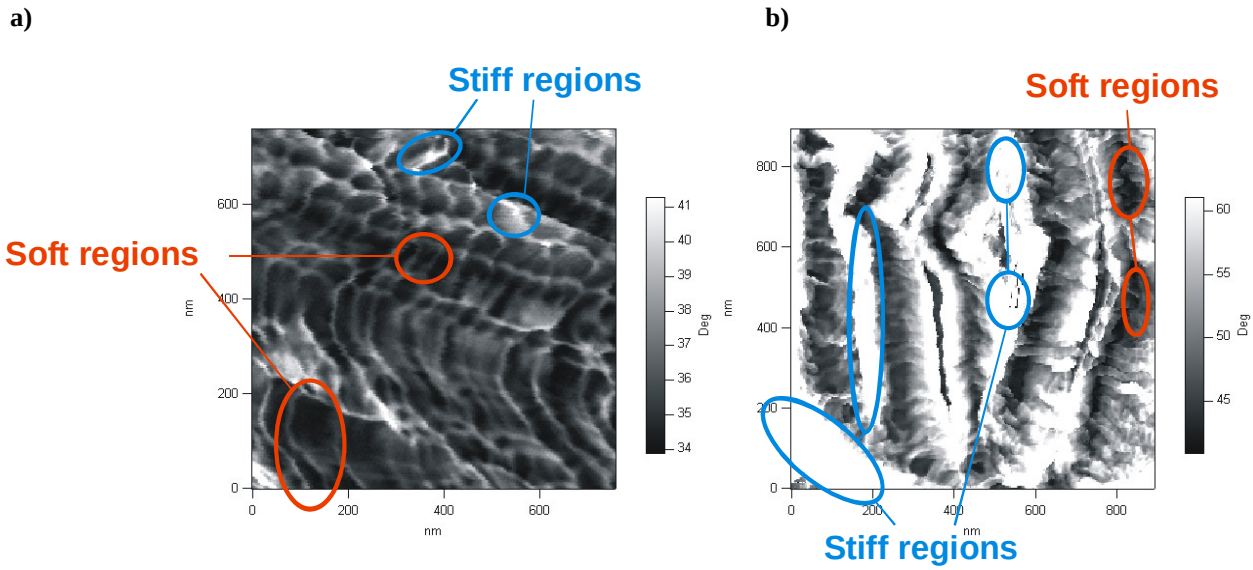


Figure 46: a) Phase Trace of a UV-sensitive endospore: scan size 667nm x 667nm; b) Phase Trace of a UV-resistant endospore: scan size 892nm x 892nm.

On each imaged spore a force mapping path is created in order to get an idea of the stiffness of the UV-sensitive and UV-resistant spores. The force mapping path includes 15 predefined points on each spore, is conducted with preset trigger forces of 3 and 12nN, respectively, and performed with 1, 2 and 4Hz (Hz refers to the number of force curves per second, indicating different pulling speeds), so that overall 2760 indentation data points are obtained.
Considering the mean indentation depth of a preset force of 3nN there are no significant differences in the penetration depth and therefore no difference in the stiffness of the spore (see Figure 47, left).
In the following table the results of the mean value of 1575 preset points of the indentation depth and the relating standard deviation of UV-sensitive and UV-resistant spores with a triggered force of 3nN and different indentation speeds are given:

UV-sensitive spores: UV-resistant spores:

Trigger point	3nN,1Hz	3nN,2Hz	3nN,4Hz	**Trigger point**	3nN,1Hz	3nN,2Hz	3nN,4Hz
Mean value of indentation depth [nm]	17,979	18,445	19,883	**Mean value of indentation depth [nm]**	15,446	16,163	17,076
Standard deviation [nm]	4,080	2,597	2,865	**Standard deviation [nm]**	5,933	7,317	6,747

Using a preset force of 12nN shows differences in the indention depth of the UV-sensitive and UV-resistant spores (see Figure 47, right). The UV-resistant spore is overall stiffer than the non-UV-resistant spore, which correlates with the obtained phase images. This stiffness could be a result of the thickness of the spore coat that is possibly larger for UV-resistant spores (higher absorption).
In the following table the results of mean value of 1185 preset points of the indentation depth and the relating standard deviation of UV-sensitive and UV-resistant spores with a triggered force of 12nN and different indentation speeds are given:

UV-sensitive spores: UV-resistant spores:

Trigger point	12nN,1Hz	12nN,2Hz	12nN,4Hz	Trigger point	12nN,1Hz	12nN,2Hz	12nN,4Hz
Mean value of indentation depth [nm]	67,511	75,904	77,364	**Mean value of indentation depth [nm]**	36,305	37,906	41,572
Standard deviation [nm]	16,215	18,699	22,750	**Standard deviation [nm]**	10,194	11,428	12,430

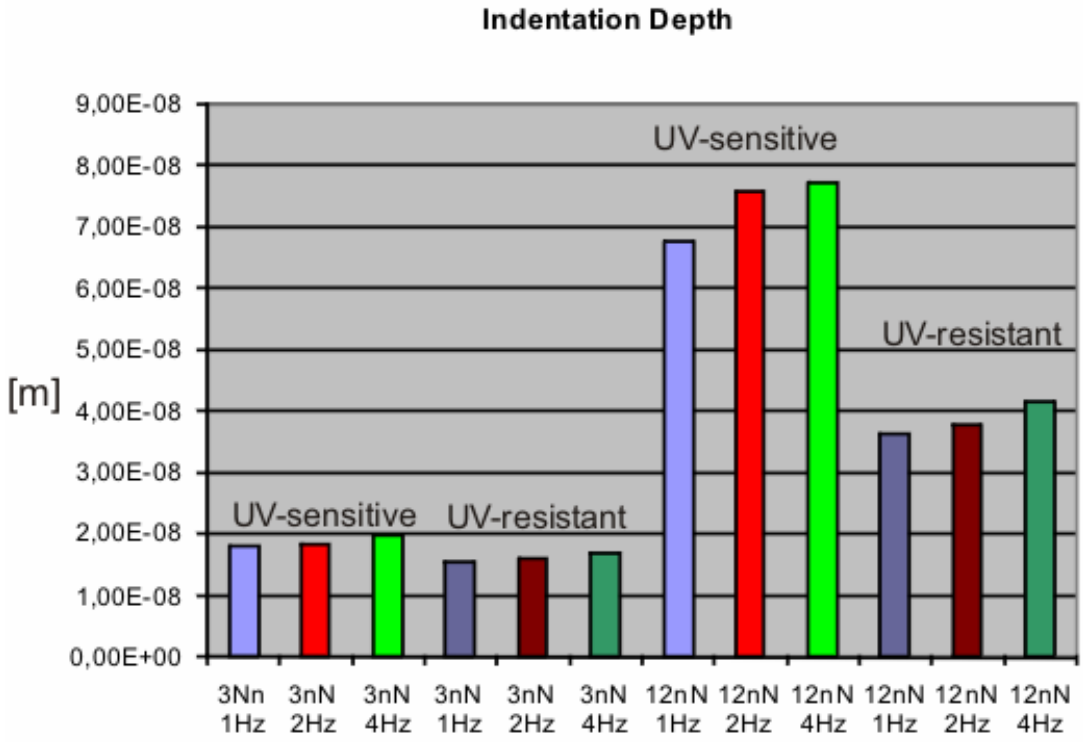


Figure 47: Diagram of the difference of the indentation depth of UV-sensitive and UV-resistant *Bacillus subtilis* spores at different indentation forces and speeds.

Conclusion, Discussion and Outlook

Investigations using the AFM have shown that it is possible to investigate biological processes and surface properties of living samples. Within this work, for the very first time, an AFM investigation has revealed the different stages of sporulation at ultrahigh resolution on vegetative *Bacillus subtilis* cells. Recording of force-distance curves and the indentation of the cantilever tip with a predefined force at certain locations on the sample surface combined with imaging on a nanoscale range provide information of cell surface structures and viscoelastic properties. The difference in the stiffness and elastic structure obtained by phase images give insight information without damaging the cells. Considering the indentation depth of the cantilever tip and therefore the stiffness of different spores by using the force mapping mode of the AFM, differences in the elasticity of UV-sensitive and UV-resistant spores are found, which might provide new information to microbiologists regarding the UV-sensitivity of *Bacillus subtilis* endospores.

Although the AFM has proved as nanobiological and nanomedical tool in the last years, it is important to mention that there are several limitations and difficulties using this technique. The main problem of imaging biological samples with the AFM is the sample preparation. The fixation of biological samples is a very important point during working with the AFM, because cell detachment can be easily caused by the scanning probe and using chemicals for fixation might lead to unintended changes of the surface structure and properties. There are still many technical problems by using the AFM for imaging biological samples. One of the main problems are the sample-probe interaction forces. By using the alternate contact mode of the AFM, the interaction forces can be reduced, but there are still damaging effects by imaging soft cell surfaces, such as animal cells[58]. Another main problem is the interpretation of and the reproduction of the obtained data. There are so many parameters, such as temperature of the room, vibrations, humidity, and many more, which have an influence on getting proper images and dates. The interpretation and understanding of the obtained data is not trivial and requires a lot of practical experience.

In the future many further investigations have to be performed, such as the development of new sample preparation techniques and instruments, the exact investigation of living cell surfaces in their ambient conditions and changes of molecular systems due to certain influences. The AFM will become a nanobiological and nanomedical tool, which will provide new structural details of very soft microbiological surfaces and will provide faster diagnosis and allow for better medications.

Appendix

Cantilever bending calculation:

E.....Elastic modulus
I.....Moment of inertia
L.....Cantilever length
F_z.....Normal Force
α.....Bending angle

The Euler equation describes the bending due to a normal force:

$$M=EI(x)\cdot\frac{d^2z}{dx^2}$$

with an expression for the moment:

$$M(x)=(L-x)\cdot F_z$$

z(x) gets:

$$z(x)=\int\int\frac{(L-x)\cdot F_z}{EI(x)}d^2x=\frac{x^2(3L-x)F_z}{6EI(x)}$$

The slope of the cantilever is:

$$z'(x)=\frac{(2Lx-x^2)F_z}{2EI}$$

For x=L and the expression $I=\frac{bh^3}{12}$ for the moment of inertia

$$z'(x)=\frac{12\cdot(2L^2-L^2)F_z}{2\cdot Ebh^3}=\frac{6\cdot L^2F_z}{Ebh^3}$$

After rearranging z(x) to

$$F_z=z(x)\cdot\frac{Ebh^3}{4L^3}$$

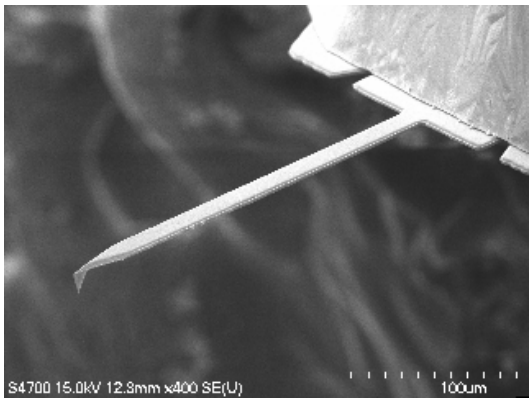
The bending angle gets

$$z'(x)=\alpha=\frac{6L^2}{Ebh^3}\cdot z(x)\cdot\frac{Ebh^3}{4L^3}=\frac{3}{2}\frac{z(x)}{L}$$

OLYMPUS OMCL-AC240TS for AC (dynamic) mode AFM

OLYMPUS OMCL-AC240TS- series cantilevers are designed for AC mode AFM in air to measure soft samples. OMCL-AC240TS- Series has a tetrahedral tip on the exact end of the cantilever.

Tetra tip cantilever, 240um long, 30um wide



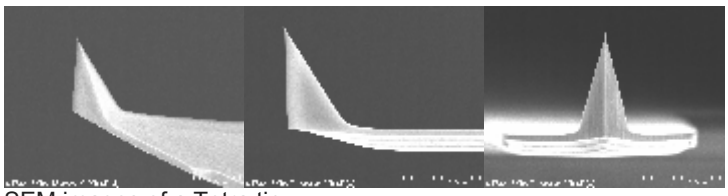
Outstanding features of Tetra tip cantilever

1) Easy to access where you want to see:
A Tetra tip is located on the very end of the cantilever. This allows you to set the tip over a point of interest on the sample, easily and precisely, if you use an AFM combined with an optical microscope.

2) High lateral resolution:
A sharper tip is required for revealing a sample surface precisely much more. Tetrahedral shape is ideal for obtaining a point terminated tip. Actually, the radius of the curvature of the Tetra tip is very small and the average is less than 10nm.
OMCL-AC240TS- series covers over wide range of applications, however, it is outstanding in the observation of soft samples like biology cells, DNA strands and so on.

Many researchers in biology and chemistry field have interests in AC mode AFM in fluid. For measuring a soft sample like a biology cell, OMCL-TR and -RC series cantilevers are recommended rather than OMCL-AC series cantilevers.

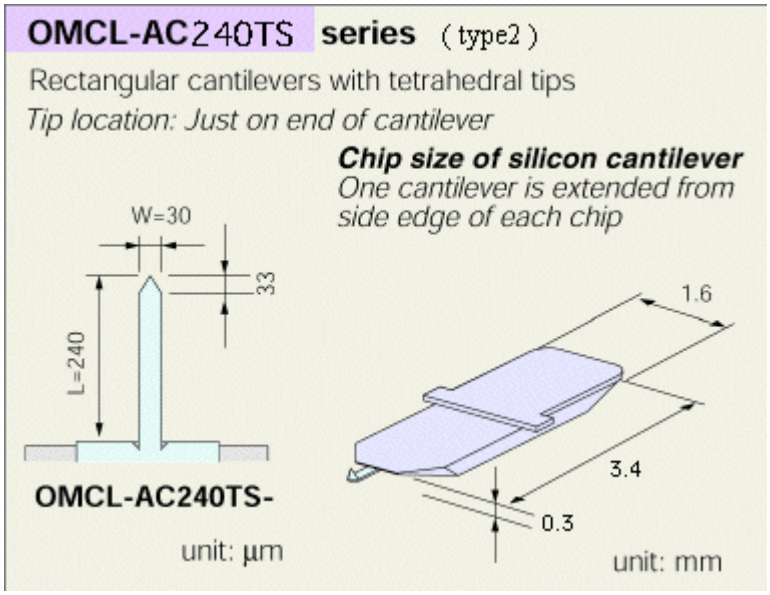
Tip:



SEM images of a Tetra tip

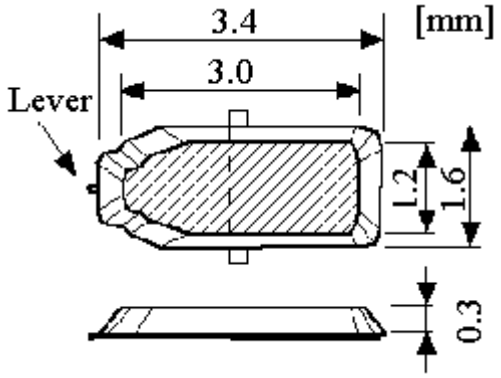
- Tip shape : sharpened tetrahedral (tilted)
  It shows good symmetry viewed from the front and is inclined viewed from the side. Considering this geometric feature, choose the fast scan (X) direction.
- Tip height : 14 micrometers (9 to 19 micrometers)
- Tip radius : smaller than 10 nm (6.8 nm (typ.))
- Tip angle : less than 35 degrees
- Tip material : single crystal silicon (semiconductor, N type, 4 - 6 ohm.cm)

Dimensions of levers and chip (substrates)



Mechanical properties of levers

Stiffness [N/m] and resonant frequency [Hz] of each cantilever are Calculated values.



	thickness (um)	length (um)	width (um)	spring const. (N/m)	resonant freq. (kHz)	height (um)	radius (nm)
OMCL-AC240TS-	2.8	240	30	1.8 (0.7 - 3.8)	70 (50 - 90)	14 (9 - 19)	< 10

Poly-L-Lysine Solution
(http://www.emsdiasum.com/microscopy/technical/datasheet/19320.aspx)

EMS Catalog #19320-A /19320-B

Intended Use:

Poly-L-Lysine solution is intended for use as an adhesive subbing solution for immunoperoxidase and routine Histologic staining preparations.

Background & Principle:

The loss of paraffin and frozen sections from slides has long been a problem during routine Histologic staining procedures. Various adhesives including albumin, gelatin and chrome alum have been applied to slides to minimize this loss. 1-3 different solutions of Poly-L-Lysine have been shown to be most effective in promoting adhesive of sections. 4-5 The polycationic nature of this molecule allows interaction with the anionic sites of tissue sections resulting in strong adhesive properties. 5. Poly-L-Lysine has been demonstrated as an effective tissue adhesive for use in various microwave procedures.

Reagent:

Poly-L-Lysine Solution. Poly-L-Lysine, 0.1% (w/v), in deionized water. Preservative added.

Precautions:

Poly-L-Lysine solution is for "In Vitro Diagnostic Use". Normal precautions exercised in handling laboratory reagents should be followed. Observe all local, state and Federal laws when disposing of waste. Refer to Material Safety Data Sheet for any updated risk, hazard or safety information. When diluted Poly-L-Lysine solution is prepared according to instructions, the maximum number of slides that can be coated is 900 per liter of diluted solution. Exceeding 900 slides per liter will affect the performance of the product.

Preparation:

Dilute Poly-L-Lysine solution 1:10 with deionized water prior to coating slides. Use plastic containers and graduated cylinders when mixing or storing solution and coating slides. Do not add fresh solution to used diluted solution.

Storage & Stability:

Dilute Poly-L-Lysine solution at room temperature (18-26°C). Reagent is stable until expiration date shown on label. Store diluted Poly-L-Lysine solution in refrigerator (2-8°C). The diluted solution is stable for at least three months. Filter diluted solution after use.

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