Light Microscope

Light Microscope

<http://en.wikipedia.org/wiki/Optical_microscope>

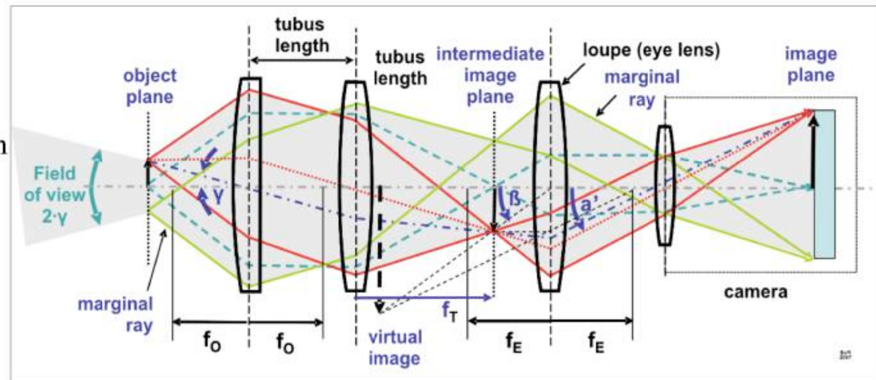
The optical components of a modern microscope are very complex and for a microscope to work well, the whole optical path has to be very accurately set up and controlled. Despite this, the basic operating principles of a microscope are quite simple.

The objective lens is, at its simplest, a very high powered magnifying glass i.e. a lens with a very short focal length. This is brought very close to the specimen being examined so that the light from the specimen comes to a focus about 160 mm inside the microscope tube. This creates an enlarged image of the subject. This image is inverted and can be seen by removing the eyepiece and placing a piece of tracing paper over the end of the tube. By carefully focusing a brightly lit specimen, a highly enlarged image can be seen. It is this real image that is viewed by the eyepiece lens that provides further enlargement.

In most microscopes, the eyepiece is a compound lens, with one component lens near the front and one near the back of the eyepiece tube. This forms an air-separated couplet. In many designs, the virtual image comes to a focus between the two lenses of the eyepiece, the first lens bringing the real image to a focus and the second lens enabling the eye to focus on the virtual image.

In all microscopes the image is intended to be viewed with the eyes focused at infinity (mind that the position of the eye in the above figure is determined by the eye's focus). Headaches and tired eyes after using a microscope are usually signs that the eye is being forced to focus at a close distance rather than at infinity.

Lighting techniques



While basic microscope technology and optics have been available for over 400 years it is much more recently that techniques in sample illumination were developed to generate the high quality images seen today.

In August 1893 August Köhler developed Köhler illumination. This method of sample illumination gives rise to extremely even lighting and overcomes many limitations of older techniques of sample illumination. Before development of Köhler illumination the image of the light source, for example a lightbulb filament, was always visible in the image of the sample.

The Nobel Prize in physics was awarded to Dutch physicist Frits Zernike in 1953 for his development of phase contrast illumination which allows imaging of transparent samples. By using interference rather than absorption of light, extremely transparent samples, such as live mammalian cells, can be imaged without having to use staining techniques. Just two years later, in 1955, Georges Nomarski published the theory for differential interference contrast microscopy, another interference-based imaging technique.

Fluorescence microscopy

Modern biological microscopy depends heavily on the development of fluorescent probes for specific structures within a cell. In contrast to normal transilluminated light microscopy, in fluorescence microscopy the sample is illuminated through the objective lens with a narrow set of wavelengths of light. This light interacts with fluorophores in the sample which then emit light of a longer wavelength. It is this emitted light which makes up the image.

Since the mid 20th century chemical fluorescent stains, such as DAPI which binds to DNA, have been used to label specific structures within the cell. More recent developments include immunofluorescence, which uses fluorescently labelled antibodies to recognise specific proteins within a sample, and fluorescent proteins like GFP which a live cell can express making it fluorescent.

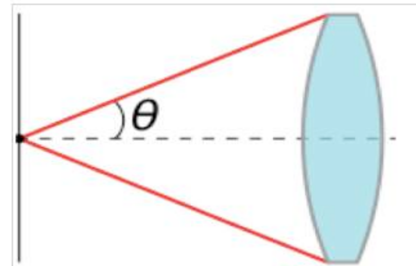
Limitations

At very high magnifications with transmitted light, point objects are seen as fuzzy discs surrounded by

diffraction rings. These are called Airy disks. The resolving power of a microscope is taken as the ability to distinguish between two closely spaced Airy disks (or, in other words the ability of the microscope to reveal adjacent structural detail as distinct and separate). It is these impacts of diffraction that limit the ability to resolve fine details. The extent and magnitude of the diffraction patterns are affected by both the wavelength of light (λ), the refractive materials used to manufacture the objective lens and the numerical aperture (NA) of the objective lens. There is therefore a finite limit beyond which it is impossible to resolve separate points in the objective field, known as the diffraction limit. Assuming that optical aberrations in the whole optical set-up are negligible, the resolution d, can be stated as: $d=\lambda/(2\,NA)$. Usually a wavelength of 550 nm is assumed, which corresponds to green light. With air as the external medium, the highest practical NA is 0.95, and with oil, up to 1.5. In practice the lowest value of d obtainable with conventional lenses is about 200 nm. A new type of lens using multiple scattering of light allowed to improve the resolution to below 100 nm.

National Model 163 Trinocular Compound Oil Immersion Microscope ($930)

Meiji inverted microscope ($4300)



Numerical aperture is a dimensionless number that characterizes the range of angles over which the system can accept or emit light: $NA=n\sin\theta$, where n is the index of refraction of the medium in which the lens is working (1.00 for air, 1.33 for pure water, and typically 1.52 for immersion oil; and θ is the half-angle of the maximum cone of light that can enter or exit the lens.

Luxo 23mm Binocular Microscope Microscope, 40X-900X System 273 ($1,610)

Amscope Phase Fluorescence Inverted ($10,000)

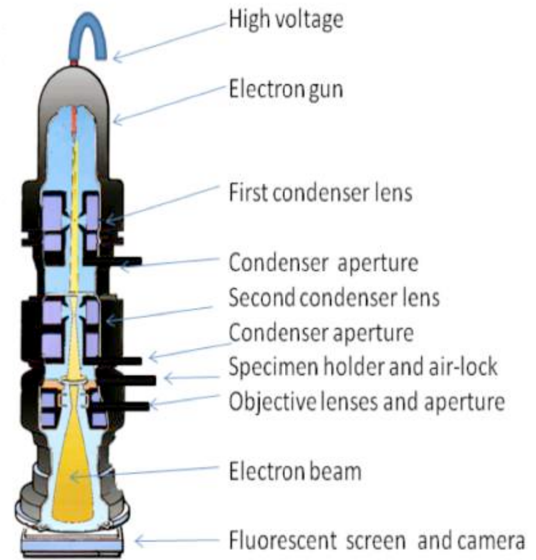
Electron Microscope

Electron Microscope

<http://en.wikipedia.org/wiki/Electron_microscope>

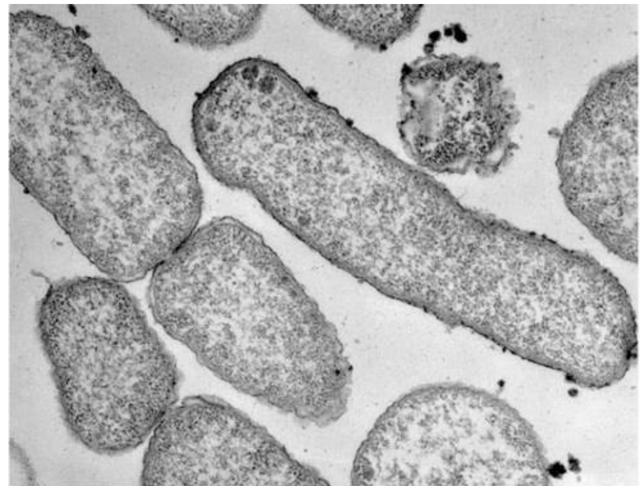
An electron microscope (EM) is a type of microscope that uses an electron beam to illuminate a specimen and produce a magnified image. An EM has greater resolving power than a light microscope and can reveal the structure of smaller objects because electrons have wavelengths about 100,000 times shorter than visible light photons. They can achieve better than 50 pm resolution[1] and magnifications of up to about 10,000,000x whereas ordinary, non-confocal light microscopes are limited by diffraction to about 200 nm resolution and useful magnifications below 2000x.

The electron microscope uses electrostatic and electromagnetic lenses to control the electron beam and focus it to form an image. These electron optical lenses are analogous to the glass lenses of a light optical microscope. Electron microscopes are used to investigate the ultrastructure of a wide range of biological and inorganic specimens including microorganisms, cells, large molecules, biopsy samples, metals, and crystals. Industrially, the electron microscope is often used for quality control and failure analysis. Modern electron microscopes produce electron micrographs, using specialized digital cameras or frame grabbers to capture the image.



Transmission Electron Microscope

Transmission electron microscope (TEM)



The original form of electron microscope, the transmission electron microscope (TEM) uses a high voltage electron beam to create an image. The electron beam is produced by an electron gun, commonly fitted with a tungsten filament cathode as the electron source. The electron beam is accelerated by an anode typically at +100 keV (40 to 400 keV) with respect to the cathode, focused by electrostatic and electromagnetic lenses, and transmitted through the specimen that is in part transparent to electrons and in part scatters them out of the beam. When it emerges from the specimen, the electron beam carries information about the structure of the specimen that is magnified by the objective lens system of the microscope. The spatial variation in this information (the "image") may be viewed by projecting the magnified electron image onto a fluorescent viewing screen coated with a phosphor or scintillator material such as zinc sulfide. Alternatively, the image can be photographically recorded by exposing a photographic film or plate directly to the electron beam, or a high-resolution phosphor may be coupled by means of a lens optical system or a fibre optic light-guide to the sensor of a CCD (charge-coupled device) camera. The image detected by the CCD may be displayed on a monitor or computer.

Resolution of the TEM is limited primarily by spherical aberration, but a new generation of aberration correctors have been able to partially overcome spherical aberration to increase resolution. Hardware correction of spherical aberration for the high-resolution transmission electron microscopy (HRTEM) has allowed the production of images with resolution below 0.5 angstrom (50 picometres) and magnifications above 50 million times.[9] The ability to determine the positions of atoms within materials has made the HRTEM an important tool for nano-technologies research and development.

An important mode of TEM utilization is electron diffraction. The advantages of electron diffraction over X-ray crystallography are that the specimen need not be a single crystal or even a polycrystalline powder,

and also that the Fourier transform reconstruction of the object's magnified structure occurs physically and thus avoids the need for solving the phase problem faced by the X-ray crystallographers after obtaining their X-ray diffraction patterns of a single crystal or polycrystalline powder. The major disadvantage of the transmission electron microscope is the need for extremely thin sections of the specimens, typically about 100 nanometers. Biological specimens typically require to be chemically fixed, dehydrated and embedded in a polymer resin to stabilize them sufficiently to allow ultrathin sectioning. Sections of biological specimens, organic polymers and similar materials may require special `staining' with heavy atom labels in order to achieve the required image contrast.

Scanning electron microscope (SEM)

Unlike the TEM, where electrons of the high voltage beam carry the image of the specimen, the electron beam of the scanning electron microscope (SEM) does not at any time carry a complete image of the specimen. The SEM produces images by probing the specimen with a focused electron beam that is scanned across a rectangular area of the specimen (raster scanning). When the electron beam interacts with the specimen, it loses energy by a variety of mechanisms. The lost energy is converted into alternative forms such as heat, emission of low-energy secondary electrons and high-energy backscattered electrons, light emission (cathodoluminescence) or X-ray emission, which provide signals carrying information about the properties of the specimen surface, such as its topography and composition. The image displayed by an SEM maps the varying intensity of any of these signals into the image in a position corresponding to the position of the beam on the specimen when the signal was generated. In the SEM image of an ant shown at right, the image was constructed from signals produced by a secondary electron detector, the normal or conventional imaging mode in most SEMs.

Generally, the image resolution of an SEM is about an order of magnitude poorer than that of a TEM. However, because the SEM image relies on surface processes rather than transmission, it is able to image bulk samples up to many centimetres in size and (depending on instrument design and settings) has a great depth of field, and so can produce images that are good representations of the three-dimensional shape of the sample. Another advantage of SEM is its variety called environmental scanning electron microscope (ESEM) can produce images of sufficient quality and resolution with the samples being wet or contained in low vacuum or gas. This greatly facilitates imaging biological samples that are unstable in the high vacuum of conventional electron microscopes.

Sample preparation

Materials to be viewed under an electron microscope may require processing to produce a suitable sample. The technique required varies depending on the specimen and the analysis required:

Chemical fixation – for biological specimens aims to stabilize the specimen's mobile macromolecular structure by chemical crosslinking of proteins with aldehydes such as formaldehyde and glutaraldehyde, and lipids with osmium tetroxide.

Negative stain – suspensions containing nanoparticles or fine biological material (such as viruses and bacteria) are briefly mixed with a dilute solution of an electron-opaque solution such as ammonium molybdate, uranyl acetate (or formate), or phosphotungstic acid. This mixture is applied to a suitably coated EM grid, blotted, then allowed to dry. Viewing of this preparation in the TEM should be carried out without delay for best results. The method is important in microbiology for fast but crude morphological identification, but can also be used as the basis for high resolution 3D reconstruction using EM tomography methodology when carbon films are used for support. Negative staining is also used for observation of nanoparticles.

Cryofixation – freezing a specimen so rapidly, in liquid ethane, and maintained at liquid nitrogen or even liquid helium temperatures, so that the water forms vitreous (non-crystalline) ice. This preserves the specimen in a snapshot of its solution state. An entire field called cryo-electron microscopy has branched from this technique.

With the development of cryo-electron microscopy of vitreous sections (CEMOVIS), it is now possible to observe samples from virtually any biological specimen close to its native state.

Dehydration – freeze drying, or replacement of water with organic solvents such as ethanol or acetone, followed by critical point drying or infiltration with embedding resins.

Embedding, biological specimens – after dehydration, tissue for observation in the transmission electron microscope is embedded so it can be sectioned ready for viewing. To do this the tissue is passed through a 'transition solvent' such as Propylene oxide (epoxypropane) and then infiltrated with an epoxy resin such as Araldite, Epon, or Durcupan; tissues may also be embedded directly in water-miscible acrylic resin. After the resin has been polymerized (hardened) the sample is thin sectioned (ultrathin sections) and stained – it is then ready for viewing.

Embedding, materials – after embedding in resin, the specimen is usually ground and polished to a mirror-like finish using ultra-fine abrasives. The polishing process must be performed carefully to minimize scratches and other polishing artifacts that reduce image quality.

Sectioning – produces thin slices of specimen, semitransparent to electrons. These can be cut on an ultramicrotome with a diamond knife to produce ultra-thin slices about 60–90 nm thick. Disposable glass knives are also used because they can be made in the lab and are much cheaper.

Staining – uses heavy metals such as lead, uranium or tungsten to scatter imaging electrons and thus give contrast between different structures, since many (especially biological) materials are nearly "transparent" to electrons (weak phase objects). In biology, specimens can be stained "en bloc" before embedding and also later after sectioning. Typically thin sections are stained for several minutes with an aqueous or alcoholic solution of uranyl acetate followed by aqueous lead citrate.

Freeze-fracture or freeze-etch – a preparation method particularly useful for examining lipid membranes and their incorporated proteins in "face on" view. The fresh tissue or cell suspension is frozen rapidly (cryofixation), then fractured by simply breaking or by using a microtome while maintained at liquid nitrogen temperature. The cold fractured surface (sometimes "etched" by increasing the temperature to about −100 °C for several minutes to let some ice sublime) is then shadowed with evaporated platinum or gold at an average angle of 45° in a high vacuum evaporator. A second coat of carbon, evaporated perpendicular to the average surface plane is often performed to improve stability of the replica coating. The specimen is returned to room temperature and pressure, then the extremely fragile "pre-shadowed" metal replica of the fracture surface is released from the underlying biological material by careful chemical digestion with acids, hypochlorite solution or SDS detergent. The still-floating replica is thoroughly washed free from residual chemicals, carefully fished up on fine grids, dried then viewed in the TEM.

Ion beam milling – thins samples until they are transparent to electrons by firing ions (typically argon) at the surface from an angle and sputtering material from the surface. A subclass of this is focused ion beam milling, where gallium ions are used to produce an electron transparent membrane in a specific region of the sample, for example through a device within a microprocessor. Ion beam milling may also be used for cross-section polishing prior to SEM analysis of materials that are difficult to prepare using mechanical polishing.

Conductive coating – an ultrathin coating of electrically conducting material, deposited either by high vacuum evaporation or by low vacuum sputter coating of the sample. This is done to prevent the accumulation of static electric fields at the specimen due to the electron irradiation required during imaging. The coating materials include gold, gold/palladium, platinum, tungsten, graphite, etc.

Earthing – to avoid electrical charge accumulation on a conductive coated sample, it is usually electrically connected to the metal sample holder. Often an electrically conductive adhesive is used for this purpose.

Disadvantages

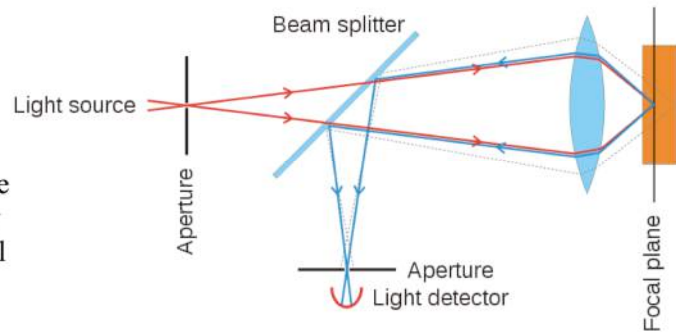
1) An electron microscope is expensive ($45K-$150K) with high installation and maintenance costs. 2) The samples largely have to be viewed in vacuum, as the molecules that make up air would scatter the electrons. 3) Scanning electron microscopes operating in conventional high-vacuum mode usually image conductive specimens; therefore non-conductive materials require conductive coating such as gold/palladium alloy, carbon, and osmium. 4) Samples of hydrated materials, including almost all biological specimens have to be prepared in various ways to stabilize them, reduce their thickness (ultrathin sectioning) and increase their electron optical contrast (staining). These processes may result in artifacts.

Confocal Microscope

Confocal Microscope

<http://en.wikipedia.org/wiki/Confocal_microscopy>

Confocal microscopy is an optical imaging technique used to increase optical resolution and contrast of a micrograph by using point illumination and a spatial pinhole to eliminate out-of-focus light in specimens that are thicker than the focal plane. It enables the reconstruction of three-dimensional structures from the obtained images. This technique has gained popularity in the scientific and industrial communities and typical applications are in life sciences, semiconductor inspection and materials science.



Basic Concept

The principle of confocal imaging was patented in 1957 by Marvin Minsky and aims to overcome some limitations of traditional wide-field fluorescence microscopes. In a conventional (i.e., wide-field) fluorescence microscope, the entire specimen is flooded evenly in light from a light source. All parts of the specimen in the optical path are excited at the same time and the resulting fluorescence is detected by the microscope's photodetector or camera including a large unfocused background part. In contrast, a confocal microscope uses point illumination (see Point Spread Function) and a pinhole in an optically conjugate plane in front of the detector to eliminate out-of-focus signal - the name "confocal" stems from this configuration. As only light produced by fluorescence very close to the focal plane can be detected, the image's optical resolution, particularly in the sample depth direction, is much better than that of wide-field microscopes. However, as much of the light from sample fluorescence is blocked at the pinhole, this increased resolution is at the cost of decreased signal intensity – so long exposures are often required.

As only one point in the sample is illuminated at a time, 2D or 3D imaging requires scanning over a regular raster (i.e., a rectangular pattern of parallel scanning lines) in the specimen. The achievable thickness of the focal plane is defined mostly by the wavelength of the used light divided by the numerical aperture of the objective lens, but also by the optical properties of the specimen. The thin optical sectioning possible makes these types of microscopes particularly good at 3D imaging and surface profiling of samples.

Three Types of Confocal Microscopes

Confocal laser scanning microscopes use multiple mirrors (typically 2 or 3 scanning linearly along the x and the y axis) to scan the laser across the sample and "descan" the image across a fixed pinhole and detector. Spinning-disk (Nipkow disk) confocal microscopes use a series of moving pinholes on a disc to scan spot of light. Programmable array microscopes (PAM) use an electronically controlled spatial light modulator (SLM) that produces a set of moving pinholes. The SLM is a device containing an array of pixels with some property (opacity, reflectivity or optical rotation) of the individual pixels that can be adjusted electronically. The SLM contains microelectromechanical mirrors or liquid crystal components. The image is usually acquired by a charge coupled device (CCD) camera.

Each of these classes of confocal microscope have particular advantages and disadvantages. Most systems are either optimized for recording speed (i.e. video capture) or high spatial resolution. Confocal laser scanning microscopes can have a programmable sampling density and very high resolutions while Nipkow and PAM use a fixed sampling density defined by the camera's resolution. Imaging frame rates are typically slower for single point laser scanning systems than spinning-disk or PAM systems. Commercial spinning-disk confocal microscopes achieve frame rates of over 50 per second – a desirable feature for dynamic observations such as live cell imaging. In practice, Nipkow and PAM allow multiple pinholes scanning the same area in parallel as long as the pinholes are sufficiently far apart. Cutting-edge development of confocal laser scanning microscopy now allows better than standard video rate (60 frames/second) imaging by using multiple microelectromechanical scanning mirrors. Confocal X-ray fluorescence imaging is a newer technique that allows control over depth, in addition to horizontal and vertical aiming, for example, when analyzing buried layers in a painting.

Atomic Force Microscope

An Introduction to Atomic Force Microscopy

N.A. Burnham, F. Cruceanu, Q. Dong and N.P. Thompson

Department of Physics, Worcester Polytechnic Institute, 100 Institute Road, Worcester, MA 01609-2280

Abstract

Atomic force microscopy (AFM) is an imaging technique used to determine topography and other properties of surfaces. It is an important tool for nanoscience.

What is a nanometer?

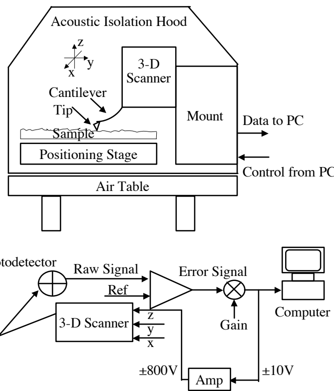
A nanometer, the unit of length associated with nanoscience, is one billionth of a meter. The ratio of a nanometer to a meter is the same as that of one millimeter to the distance between Worcester and Detroit.

Map from US CIA

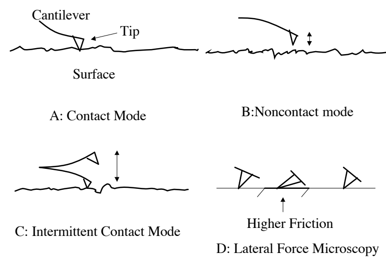
Length scales

Diameter of human hair	100 μm	Largest scan range of our AFM
Practical limit of optical microscopy	10 μm	Typical scan range of our AFM
Distance between CD tracks	1 μm	Smallest practical scan range
Wavelength of ultraviolet light	100 nm	Typical tip radius
Diameter of backbone of DNA	10 nm	Typical tip-sample contact radius
Length of napthalene molecule	1 nm	Lattice resolution
Diameter of hydrogen atom	0.1 nm	Atomic resolution

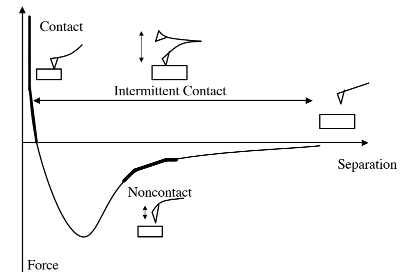
How does AFM work?



Above – our AFM setup. A photodetector records the bending of the cantilever, which reflects the topography and other surface properties. The cantilever is a flexible beam and is used as a force sensor. It is located on the scanner which can move in three dimensions.
Below – the photodetection and feedback system, which works to keep the tip-sample force steady according to Hooke's Law, F = -kd.



There are four principal kinds of **operational modes** according to the separation between the cantilever and the surface, the cantilever's oscillation amplitude, and the normal or torsional bending of the cantilever. Many other variations also exist.



Schematic of a typical **force-separation curve**. Far from the sample, the cantilever is not affected by interatomic forces and is in its free equilibrium position. But when brought closer to the surface, attractive forces act upon the tip to bend the cantilever towards the sample. When the tip is in contact with the surface, repulsive forces dominate, deflecting the cantilever backwards. The thick lines indicate the normal ranges of operation for contact and noncontact modes and the long horizontal arrow represents the usual amplitude for intermittent contact.

Advantages and disadvantages of AFM

- \+ Easy sample preparation
- \+ Accurate height information
- \+ Works in vacuum, air, and liquids
- \+ Living systems can be studied

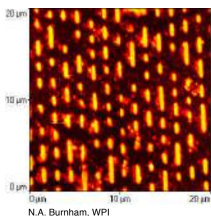
- – Limited vertical range
- – Limited magnification range
- – Data not independent of tip
- – Tip or sample can be damaged

Ways of using AFM

Cost: ~$20K – ~$1M

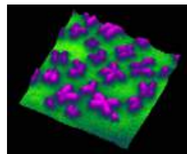
- Imaging – determining the topography of the surface
- Measuring – characterizing the sample's materials properties
- Manipulating – purposefully changing the surface structure
- Sensing – using AFM technology for sensor applications

What is AFM used for?



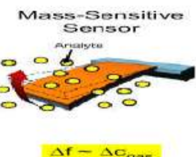
N.A. Burnham, WPI

Imaging - The AFM can be used to construct a picture of a CD stamper (left) or human chromosomes (right).

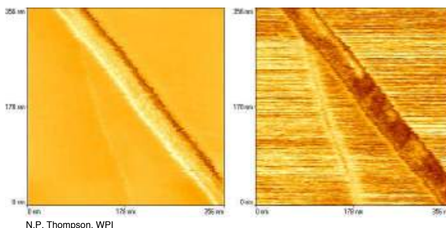


http://spm.phy.bris.ac.uk/people/TerryMcMaster

Sensing - A microcantilever bends in response to chemical adsorption. Based on this principle, AFM is used to "smell" different substances (such as whisky) in order to establish their composition.

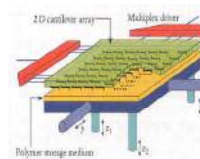


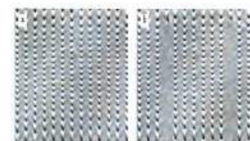
Oliver Brand, www.iqe.ethz.ch, Nature **414** (2001)



N.P. Thompson, WPI

Measuring - Carbon nanotubes are rolled-up sheets of graphite and have valuable electronic and mechanical properties. The left image reflects the topography of a nanotube near a step on a mica surface. To create the image on the right, the AFM scanner oscillated laterally a small amount and the resulting oscillation of the cantilever was monitored, reflecting the amount of shear between the tip and the sample.





www.Zurich.ibm.com, Physics Today, October 2002

Manipulating - Using 1024 cantilevers, the "Millipede" can write on a surface of polymer film. The actuators (dark blue) move the polymer-coated substrate in the xy-plane beneath the cantilever array. Each cantilever is individually controlled and can produce or erase a small pit that represents a bit of information. It is predicted that 100 GB of memory can be put on only one square inch using this technology.