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ARTICLE

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MICROSCOPY: A NEW LOOK AT A VERY OLD TECHNIQUE

ALOKMAY DATTA*

This very brief review seeks to introduce the basics of microscopy and of far-field and nearfield microscopies, with some suitable examples of both types of microscopes that are frequently used in laboratories. Specifically, it outlines the working principles of Confocal Microscopes, Brewster-Angle Microscopes, Electron Microscopes, Atomic Force Microscopes and Near-field Optical Microscopes.

What is Microscopy?

Microscopy is at the same time a very old and a very young technique. While its beginnings go back to the quartz magnifying lenses used around 750 B.C. by Assyrian jewellers in Nimrud[1], the latest are the lens less transmission electron microscopes that have been perfected over the last couple of years to provide a view of the insides of single atoms[2].

Microscopy means creating a response $I(x,y,z)$ from an object and then enhancing this response either by a factor M, i.e., then the final response is $MI(x,y,z)$ or by another spacedependent function $F(x,y,z)$, when the final output is $F(x,y,z)I(x,y,z)$. $I(x,y,z)$ is then called the initial image of the object, M is called the magnification, $F(x,y,z)$ is called the filtered magnification and $MI(x,y,z)$ or $F(x,y,z)I(x,y,z)$ is called the final image.

The most important parameter or quantity in a microscope is its *resolution*. It is given in the x, y and z directions by Δx, Δy and Δz, respectively where these are the minimum lengths for which $(dI/dx)\Delta x$, $(dI/dy)\Delta y$, and $(dI/dz)\Delta z$ are large enough to be detected. Hence, resolution is *completely determined* by the nature of $I(x,y,z)$, i.e., how it is obtained or the physical quantity being measured, the type of sample, the *kind of detector* and the *geometry of the detector-sample system*. Resolution is *not* affected by magnification.

There are two fundamentally different types of microscopes – *far-field* and *near-field*. The terms 'far' and 'near' refer to some characteristic length such as the wavelength of the light used. If the response-gathering element is placed at a distance smaller than this characteristic length from the sample it is called a near-field microscope, otherwise it is a farfield microscope. In the first category we have the *optical microscopes* and *electron microscopes* using lenses. In the second category we have the *scanning probe microscopes* and the *scanning optical microscopes*, where we place a *probe* very near the sample and scan either the sample or the probe.

Far-field Microscopy: Basics

In optical or electron far-field microscopes the response of image I is the transmittance T or the reflectance

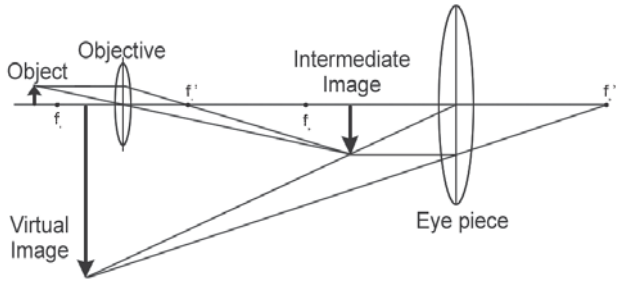


Fig. 1. General Layout of a Far-field Microscope

* Department of Physics, University of Calcutta, 92, A.P.C. Road, Kolkata 700 009, India

e-mail: alokmaydatta@gmail.com

R of photons or electrons. In most modern optical microscopes both *T* and *R* can be used but in *Transmission Electron Microscopy* (TEM) only *T* and in *Scanning Electron Microscopy* (SEM) only *R* is used because for electrons these fall in two different energy ranges, high for the former and low for the latter.

In both modes and for both photons and electrons, the image is created by a lens with a small focal length *f*, and the object is placed between distances *f* and 2*f* from this lens. A magnified real image $I_o(x,y)$ is formed behind the lens at a distance greater than 2*f*. a second lens, with a larger focal length *f´* is placed at the back of the first lens ,such that $I_o(x,y)$ becomes the 'object' for the second lens, and is placed between *f´* and this lens. This 'object' then forms a virtual and very much magnified image $I_e(x,y)$ in front of the first lens. The situation is depicted in Figure 1. The first lens is called the *objective* and the second is called *eyepiece*. For electron microscopes as well as modern optical microscopes the second image is a magnified real image, so that it can be recorded by a camera on an image plate. Magnification of objective is *m* and that of eye-piece is *m´*, hence total magnification is $M = mm'$.

Optical Microscopes in Recent Use

Confocal Microscope : In traditional optical microscopes, the entire specimen is subjected to intense illumination from an incoherent mercury or xenon arc-discharge lamp, and the resulting image of reflection, transmission or fluorescence emission from the specimen can be viewed directly in the eyepieces or projected onto the surface of an electronic array detector or traditional film plane[3]. In contrast to this simple concept, the confocal microscope is a *scanning microscope* consisting of multiple laser excitation sources, a scan head with optical and electronic components, electronic detectors (usually photomultipliers), and a computer for acquisition, processing, analysis, and display of images[4].

Confocal microscopy offers several advantages over conventional optical microscopy, including the ability to control depth of field, elimination or reduction of background information away from the focal plane (that leads to image degradation), and the capability to collect serial optical sections from thick specimens. The key to the confocal approach is the use of spatial filtering techniques *to eliminate out-of-focus light or glare in specimens whose thickness exceeds the immediate plane of focus.*

The confocal principle in laser scanning microscopy is presented in Figure 2. Coherent light emitted by the laser system (*excitation source*) passes through a pinhole aperture that is situated in a *conjugate plane (confocal)* with a scanning point on the specimen and a second pinhole aperture positioned in front of a *photomultiplier tube* (the detector)[5]. As the laser is reflected by a *dichromatic mirror* and scanned across the specimen in *a defined focal plane*, lighted reflected or emitted from points on the specimen (in the same focal plane) pass back through the dichromatic mirror and are focused as a *confocal point* at the detector pinhole aperture.

The significant amount of reflection or emission that occurs at points above and below the objective focal plane is not confocal with the pinhole (termed Out-of-Focus Light Rays in Figure 2). These point objects are seen as hazy discs surrounded by diffraction rings, the discs being known as *Airy discs*. These extended Airy discs are formed in the aperture plane. Because only a small fraction of the out-of-focus light is delivered through the pinhole aperture, most of this light is not detected by the photomultiplier and *does not contribute to the resulting image*.

Refocusing the objective in a confocal microscope shifts the excitation and emission points on a specimen to a new plane that becomes confocal with the pinhole apertures of the light source and detector.

Brewster-Angle Microscope

Unpolarized light incident on a surface can be taken to be a superposition of equal *s* (perpendicular to surface) and *p* (parallel to surface) polarized light. Thus the total reflectance for unpolarized light is $(R_s + R_p)/2$. The reflectivity for p-polarized light goes to zero at a particular angle (around 56° for water surface), known as Brewster's angle. If an unpolarized light is incident at the Brewster's angle, the reflected light is purely s-polarized. No light is reflected from the interface for the incidence of p-polarized light at the Brewster's angle. If a transparent sample with a different refractive index is at this interface, its Brewster angle being different, it will reflect light to the detector and the intensity of this reflected light will be proportional to the total dipole moment of the illuminated volume of the sample. For an uniform medium it will be proportional to the sample thickness, while the variation in the dipolar density in a sample is given by the variation of this intensity if the thickness is either kept fixed or determined by some other means. This is the basis for Brewster angle microscopy[7]. A schematic diagram is shown in Figure 3.

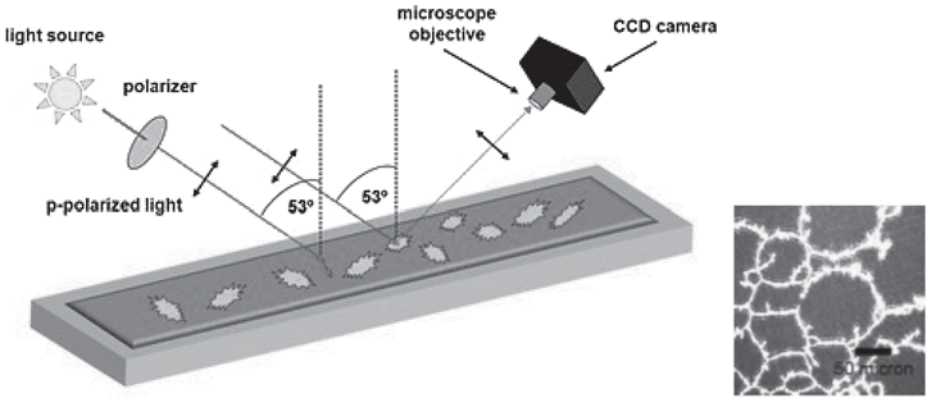


Fig. 3. (a) Schematic diagram of the Brewster Angle Microscope [ref 7]; (b) Brewster angle microscopy image of Au nanoparticle-stearic acid mixed monolayer on water [ref. 8].

We can find the Brewster's angle (θ_B) for two media by setting $R_p = 0$ and $\theta_1 = \theta_B$, i.e,

$$n_2 \cos\theta_B = n_1 \cos\theta_2$$

Using Snell's law, $\left(n_1 \sin\theta_B = n_2 \sin\theta_2\right)$ in the above equation and substituting n_1/n_2, we have the following relation,

$$\sin 2\theta_B = \sin 2\theta_2$$

To hold the above equality it requires that $\theta_B + \theta_2 = \pi/2$. This signifies that the refracted ray is perpendicular to the reflected ray when the p-polarized light is incident at the Brewster's angle. Dividing Snell's law by the first equation, one can have the following relation,

$$\frac{n_1}{n_2}\tan\theta_B = \frac{n_2}{n_1}\tan\theta_2$$

Now, using the relation, $\theta_B + \theta_2 = \pi/2$ in the above equation, we get the Brewster's angle in terms of refractive indices,

$$\theta_B = \tan^{-1}\frac{n_2}{n_1}$$

The above relation holds when the media are semi-infinite and non-absorptive.

Electron Microscopes

Scanning Electron Microscope : When electrons are accelerated up to high energy levels (few hundred keV) and focused on a material, they can scatter or backscatter elastically or inelastically, or produce many interactions, source of different signals such as X-rays, Auger electrons or light (Figure 4(a))[9]. Some of them are used in electron microscopy (EM).

The Scanning Electron Microscope (Figure 4(b)) images the sample surface by scanning it with a high-energy beam of electrons in a raster scan pattern. The electrons interact with the atoms that make up the sample producing mainly secondary electron signals that contain information about the sample's surface topography, composition and other properties such as electrical conductivity.

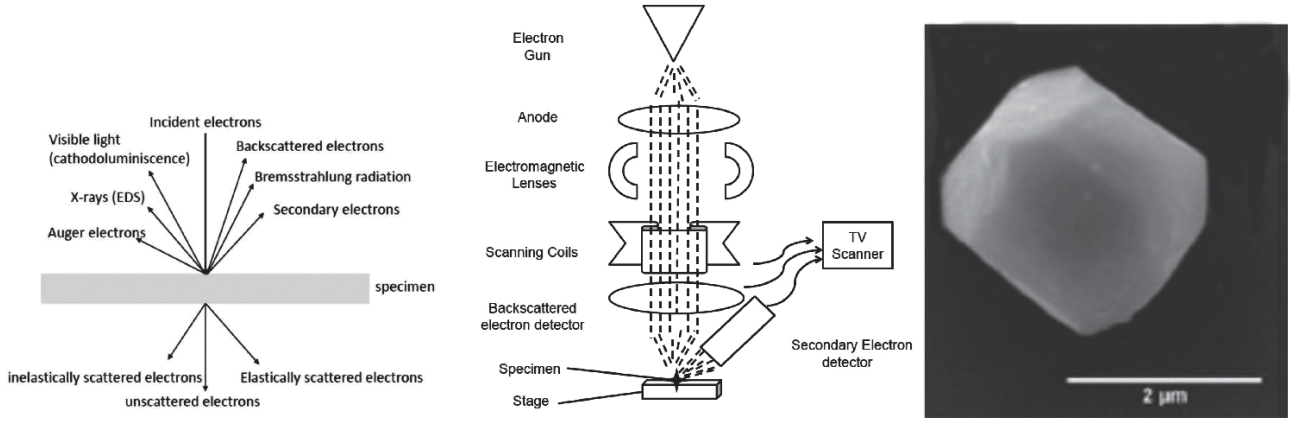
Transmission Electron Microscope

X-rays or γ-rays have lower wavelength than visible light, but unfortunately, high performance lenses necessary to focus the beam to form an image are difficult to achieve. However, as the de Broglie wavelength associated with electrons is given by $\lambda = h/p$, where p is the relativistic momentum and h the Planck's constant, we can control the wavelength to atomic length scales by changing the electron momentum through its energy, while electric and magnetic fields can easily be used as 'electron lenses'. In a TEM (Figure 4), electrons are accelerated to a kinetic energy of 100 to 1000 keV. The associated wavelength is five orders of magnitude smaller than the light wavelength (0.1-0.01 nm). This resolution enables material imaging and structure determination at the atomic level[10].

Modes of Transmission Electron Microscopy

Imaging Mode : A transmission electron microscope is constituted of: (1) two or three condenser lenses to focus the electron beam on the sample, (2) an objective lens to form the diffraction in the back focal plane and the image of the sample in the image plane, (3) some intermediate lenses to magnify the image or the diffraction pattern on the screen. If the sample is thin (< 200 nm) and constituted of light chemical elements, the image presents a very low contrast when it is focused. This set-up is shown schematically in Figure 5(a).

Diffraction Mode : The selected area diaphragm is used to select only one part of the imaged sample for



(a) (b) (c)

Fig. 4. (a) Different electron-matter interactions [ref. 9]; (b) Schematic of a Scanning Electron Microscope [ref. 10]; (c) Scanning electron microscopy image of Bombyx mori nucleopolyhedrovirus [ref. 11]

example a particle or a precipitate. This mode is called selected area diffraction (SAED) mode (Figure 5(b)). The spherical aberrations of the objective lens limit the area of the selected object to few hundred nanometers. Nevertheless, it is possible to obtain diffraction patterns of a smaller object by focusing the electron beam with the projector lenses to obtain a small spot size on the object surface (2-10 nm). The spots of SAED become disks whose radii depend on the condenser diaphragm. This is called micro-diffraction. SAED and micro-diffraction patterns of a crystal permit to obtain the symmetry of its lattice and calculate its interplanar distances. This is useful to confirm the identification of a phase, after assumptions generally based on the literature of the studied system and on chemical analyses.

The Basic Problem of Resolution in Far-field Microscopes

In a far-field microscope the resolution is the distance between two adjacent Airy discs. This distance is decided by the wavelength λ of the light, the refractive indices of the lenses with respect to the surrounding media and the numerical aperture of the objective lens, as expressed by Abbé's relation

$$d = \lambda/(2\,\mu \sin \alpha_0)$$

where, d is the minimum resolvable distance, μ is the refractive index of the objective relative to the surrounding medium, α_0 is the angular aperture of the objective, i.e., the angle spanned by the objective as seen from the sample. For an oil-immersed objective at maximum aperture, $\sin \alpha_0 = 1$ and $\mu = 1.5$. Hence, $d = \lambda/3$. For white light,

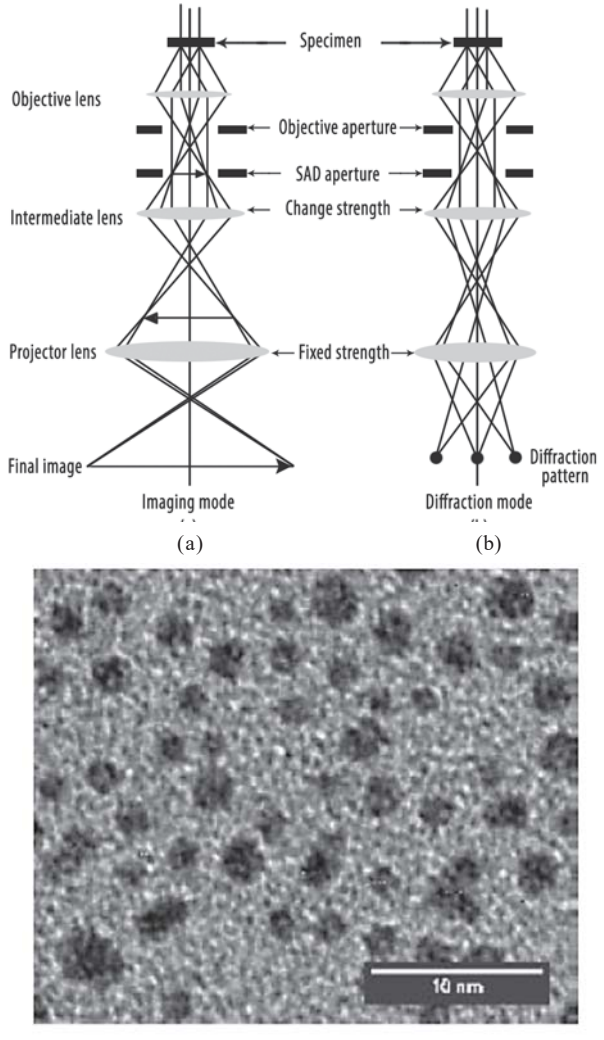


Fig. 5. Schematic of a Transmission Electron Microscope in (a) Imaging Mode and (b) Diffraction Mode [ref. 12]; (c) Transmission electron microscopy image of dodecanethiolcapped Au nanoparticles [ref.13]

since median or optimum wavelength ~ 600 nm, $d \sim 200$ nm. This is the best resolution obtainable in conventional optical microscopes.

Various recently introduced laser-optical 'nanoscopy' approaches permit to overcome this problem, and to extend the spatial analysis beyond the Abbé limit. They can reach optical resolution to $\lambda/2$ in the object plane (x, y) and to λ in the z-axis. The above techniques rely on the difference in the optical signals obtained from the different parts of the sample for some specific exciting radiation. They are critically dependent on (1) the monochromaticity and small beam size of the exciting light on one hand and (2) the frequency selectivity and sensitivity of the detector[14].

An example of such super resolution optical microscope is the 4Pi-Single Molecule Switching (4Pi-SMS) Microscope developed by Hell[16] for which he received Nobel Prize in Chemistry in 2014. This is an interference-based method, where fluorescence emitted by a single molecule i.e., a fluorophore, is collected by two opposing objective lenses with a common focus. It then follows two identical, but separate, beam paths and finally combined with a beam splitter to cause interference. Any two adjacent points on the molecule will travel through different paths and will produce their own interference pattern. The overall pattern will be a superposition of all the two-point patterns and the molecular shape can be reconstructed from a Fourier transform of the final pattern. The resolution is thus decided by the resolution of the transform and can be enhanced by a factor of 7 over the radiation wavelength. The ideal solid angular aperture of an objective is 2π, so with two objectives a total of 4π solid angle is covered, i.e. the sample is probed from all sides. Hence the reconstruction is three-dimensional.

Near-field or Scanning Probe Microscopy

Even with high performance lasers and detectors the resolution is never better than 5 nm. The general principle to overcome this limitation is to create an image through a physical property that changes significantly within a much smaller length scale, or in our terminology, a faster spatial response. One way to achieve this, as we have seen, was to use 'light' or 'particles' with smaller wavelengths to probe properties such as electron densities that changed with that kind of fields. However, that requires special treatment and conditions of the samples, namely, ultrahigh vacuum and thinning the samples to make them 'transparent' to the radiations, which take them far away from pristine or natural states and thus may not be always preferred.

Scanning Probe Microscopy (SPM) is a branch of microscopy that forms images of surfaces using a physical probe that scans the specimen. An image of the surface is obtained by mechanically moving the probe in a raster scan of the specimen, line by line, and recording the probe-surface interaction as a function of position. SPM was founded with the invention of the scanning tunneling microscope in 1981[17]. Many scanning probe microscopes can image several interactions simultaneously. The manner of using these interactions to obtain an image is generally called a mode.

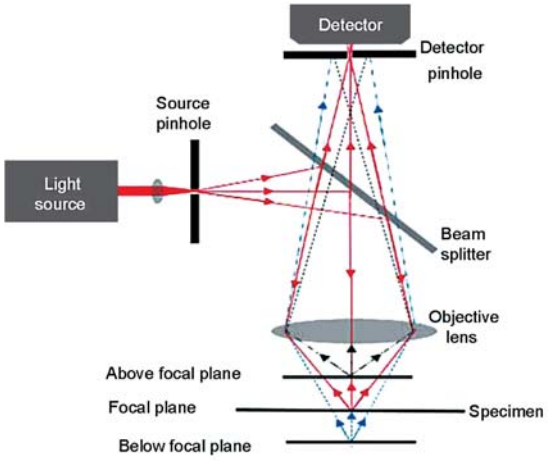
The resolution varies somewhat from technique to technique, but some probe techniques reach a rather impressive atomic resolution. They owe this largely to the ability of piezoelectric transducer to execute motions with a precision and accuracy at the atomic level or better on electronic command. One could rightly call this family of techniques 'piezoelectric techniques'. The other common denominator is that the data are typically obtained as a two-dimensional grid of data points, visualized in false colour as a computer image.

The major advantage is that resolution of the microscopes is not limited by diffraction, i.e. Abbé's criterion but only by the size of the probe-sample interaction volume (i.e., point spread function), which can be as small as a few pm. Hence the ability to measure small local differences in object height (like that of 135 pm steps on < 100 > silicon) is unparalleled. Laterally the probe-sample interaction extends only across the tip atom or atoms involved in the interaction. The second advantage is that this probe-sample interaction can be used to modify the sample to create small structures[20]. This is known as *nanolithography*. Lastly, unlike electron microscope methods, specimens do not require a partial vacuum but can be observed in ambient or in many prescribed conditions.

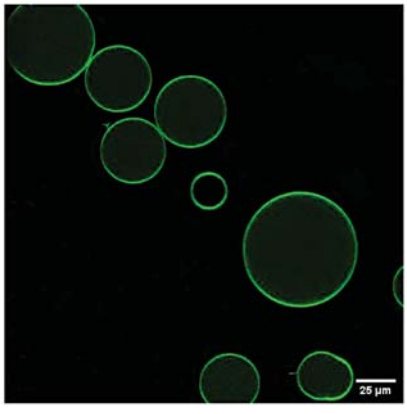
We now look at two scanning probe microscopes commonly used for probing soft matter – *Atomic Force Microscope* (AFM) and *Near-field Scanning Optical Microscope* (NSOM).

Atomic Force Microscope

The *atomic force microscope* (AFM) or *scanning force microscope* (SFM) was invented in 1986 by Binnig,



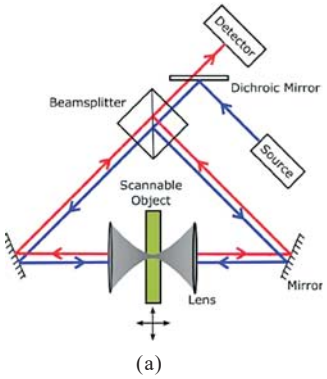
(a)



(b)

Fig. 2. (a) Schematic of the Confocal Microscope [ref. 5]: (b) Confocal microscopy image of 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC) liposomes [ref. 6]

Quate and Gerber. The AFM raster scans a sharp probe over the surface of a sample and measures the changes in force between the probe tip and the sample18,20. Figure 7(a) illustrates the working concept for an atomic force microscope. A cantilever with a sharp tip is positioned above a surface. Depending on this separation distance,



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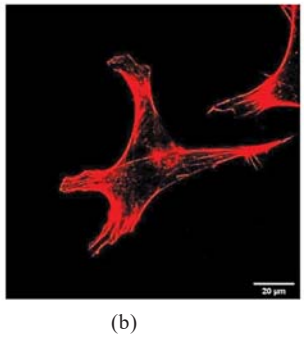
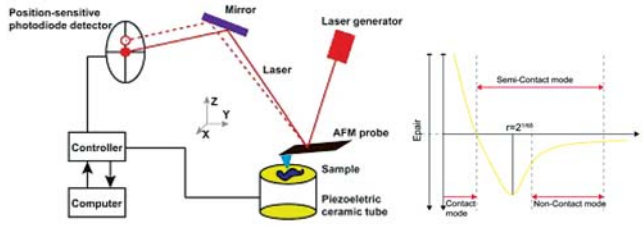
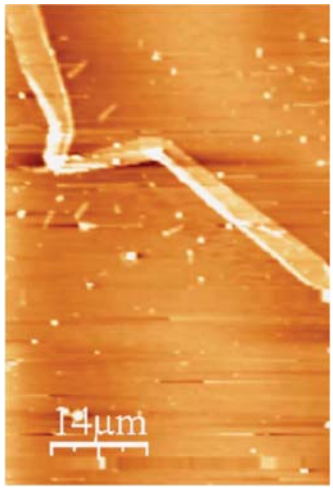


(b)

Fig. 6 (a) Schematic of a 4Pi-Single Molecule Switching (4Pi-SMS) Microscope [ref 15]; (b) Images of actin filaments [ref 6]

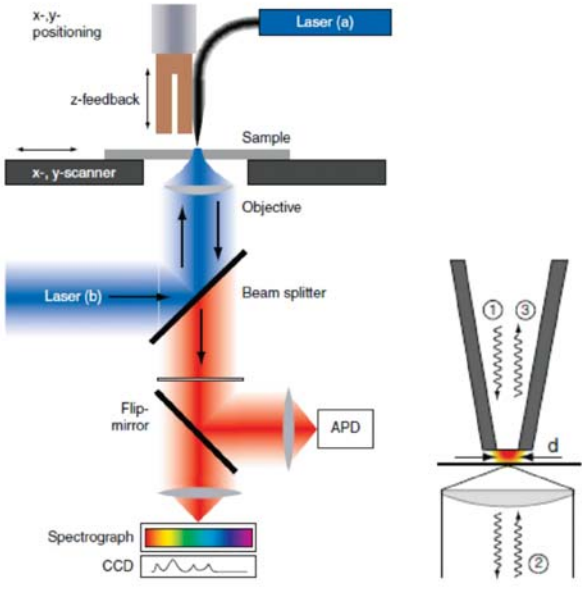


(a) (b)



(c)

Fig. 7. (a) Schematic of an Atomic Force Microscope and (b) the different modes on the interaction potential [ref. 18]; (c) Tapping mode atomic force microscopy image of Cadmium Stearate monolayer collapsed on water surface and transferred onto Si substrate [ref. 19].



(a) (b)

Figure 8. (a) Schematic Diagram and (b) modes of operation of aperture-Near-field Scanning Optical Microscope [ref. 22]. Near-field excitation–far-field collection (1) far-field excitation – near-field collection (2) and near-field excitation – collection mode (3).

long range or short range forces will dominate the interaction as shown in Figure 7(b). This force is measured by the bending of the cantilever by an optical lever technique: a laser beam is focused on the back of a cantilever and reflected into a photo-detector. Small forces between the tip and sample will cause less deflection than large forces. By raster-scanning the tip across the surface and recording the change in force as a function of position, a map of surface topography and other properties can be generated.

AFM is useful for obtaining three-dimensional topographic information of insulating and conducting structures with lateral resolution down to 1.5 nm and vertical resolution down to 0.05 nm. These samples include clusters of atoms and molecules, individual macromolecules, and biological species (cells, DNA, proteins). AFM can operate in gas, ambient, and fluid environments and can measure physical properties including elasticity, adhesion, hardness, friction and chemical functionality.

The three general types of AFM imaging are (1) contact mode, (2) tapping mode and (3) noncontact mode.

Contact mode is the most common method of operation of the AFM and is useful for obtaining 3D topographical information on nanostructures and surfaces. As the name suggests, the tip and sample remain in close contact as the scanning proceeds. "Contact" represents the repulsive regime of inter- molecular force curve, the part of the curve above the x-axis in Figure 7(b). Most cantilevers have spring constants < 1 Nm, which is less than effective spring constant holding atoms together. One of the drawbacks of the tip remaining in contact with the sample is that large lateral forces can be exerted on the sample as the tip is dragged over the specimen. These large forces can result in deformed images and damaged samples. Small lateral forces, however, can be used to provide information on the friction (drag resistance) between the tip and sample in a mode known as lateral force microscopy (LFM). LFM measures the torsional deformation of the cantilever while the tip scans over the surface.

Tapping mode is another mode of operation for AFM. Unlike the operation of contact mode, where the tip is in constant contact with the surface, in tapping mode the tip makes intermittent contact with the surface. As the tip is scanned over the surface, the cantilever is driven at its resonant frequency. Because the contact time is a small fraction of its oscillation period, the lateral forces are reduced dramatically. Tapping mode is usually preferred to image samples with structures that are weakly bound to the surface or samples that are soft (polymers, thin films). There are also two other types of image contrast mechanisms in tapping mode:

Amplitude imaging: The feedback loop adjusts the z-piezo so that the amplitude of the cantilever oscillation remains (nearly) constant. The voltages needed to keep the amplitude constant can be compiled into an (error signal) image, and this imaging can often provide high contrast between features on the surface.

Phase imaging: The phase difference between the driven oscillations of the cantilever and the measured oscillations can be attributed to different material properties. For example, the relative amount of phase lag between the freely oscillating cantilever and the detected signal can provide qualitative information about the differences in chemical composition, adhesion, and friction properties.

Non-contact mode is a method where the cantilever is oscillated above the surface of the sample at distance such that it is no longer in the repulsive regime but in the attractive regime of the inter- molecular force curve.

The choice for which AFM mode to use is based on the surface characteristics of interest and on the hardness/ stickiness of the sample. Contact mode is most useful for hard surfaces; a tip in contact with a surface, however, is subject to contamination from removable material on the surface. Excessive force in contact mode can also damage the surface or blunt the probe tip. Tapping mode is well-suited for imaging soft biological specimen and for samples with poor surface adhesion (DNA and carbon nanotubes). Non-contact mode is another useful mode for imaging soft surfaces, but its sensitivity to external vibrations and the inherent water layer on samples in ambient conditions often causes problems in the engagement and retraction of the tip.

Near-field Scanning Optical Microscope

The Abbé criterion only assumes the light diffracted into the far-field that propagates without any restrictions. NSOM makes use of evanescent or non-propagating fields that exist only near the surface of the object. These fields carry the high frequency spatial information about the object and have intensities that drop off exponentially with distance from the object. Because of this, the detector must be placed very close to the sample in the near field

zone, typically a few nm. As a result, near field microscopy remains primarily a surface inspection technique. The detector is then rastered across the sample using a piezoelectric stage. The scanning can either be done at a constant height or with regulated height by using a feedback mechanism.

In the most widely implemented form of this microscopy (known as aperture-NSOM) the sample is illuminated through a small aperture held in close proximity (at a distance less than 10 nm) to the sample surface (Figure 8(a)). The aperture can operate either as an emitter (that is, as a nanoscopic light source to illuminate the sample) or as a receiver (to collect light from the sample), or both as shown in Figure 8(b)[22].

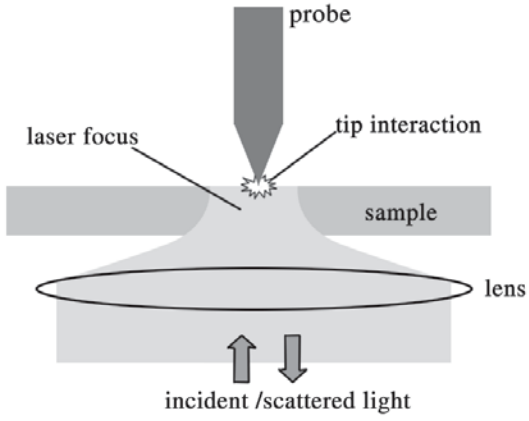


Fig. 9. Schematic for Apertureless-NSOM [ref. 24]

'Apertureless' NSOM techniques are so-called because they do not rely on the illumination of the sample through a small aperture. Apertureless NSOM may involve local scattering of light from a sample surface, local enhancement of an optical signal, or local fluorescence (for example, light emission from a single molecule attached to a scanning tip)21. A scheme for the realization of Apertureless NSOM is illustrated in Figure 8.

As well as providing nanometer resolution optical microscopy of surfaces, a powerful further feature of NSOM results from the requirement for the NSOM probe (whether this is an aperture or otherwise) to be held a short distance above the sample surface at all times. In this way, sample topography is mapped simultaneously with the formation of the optical image of the sample as the tip is scanned over the sample surface, allowing a correlation between optical properties and topography and enabling a comparison with the more mature technique of AFM. Furthermore, though it is a surface sensitive technique yet it is also an optical technique. Hence NSOM (and aperture-NSOM in particular) is able to probe systems beneath the surface of a transparent material.

Outlook

A major problem in microscopy is the mutual exclusivity of resolution and field of view. In the process of probing the minute details of a part of the specimen, almost all information of the perspective, i.e. the morphological connection of that part with other parts, is lost. On the other hand, a perspective view debars us from obtaining any further details. While this, unfortunately, is a fundamental barrier, a close approximation to its mitigation lies in acquiring a series of images at a high resolution while moving the sample slowly across the field of view, keeping the image collection process optimized throughout. Such a technique will also open up a new area of microscopy where a sample can be studied at high resolution while its environment is being changed, thus initiating research on system-environment interaction on a new footing. ❐

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