2. Optical Microscopy

2.1 Principles

A microscope is in principle nothing else than a simple lens system for magnifying small objects. The first lens, called the objective, has a short focal length (a few mm), and creates an image of the object in the intermediate image plane. This image in turn can be looked at with another lens, the eye piece, which can provide further magnification.

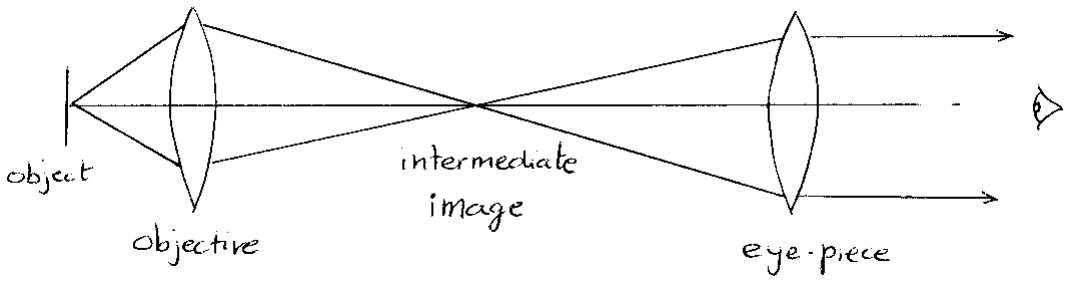

Figure 2.1 : Principle scheme of an optical microscope. The objective lens has a much shorter focal length than the eye-piece, in order to magnify the intermediate image (usually by a factor 40-100).

The resolution of the image is limited by diffraction. The Abbe-Rayleigh criterion states that, for a wavelength λ, the smallest distance $d_{\min}$ resolvable between two point sources in the object plane, as deduced from diffraction theory, is :

$$d_{\min} = 1.22 \times \frac{\lambda}{2NA} \quad ,$$

where $NA = n \times \sin\alpha$ is called *numerical aperture* of the objective lens. *n* is the index of refraction in the *object* space, and α half the maximal angle under which the objective lens collects light from the object. This relation is equivalent to a Fourier (or Heisenberg) relation applied to space and transverse wavevector $K = n\frac{\omega}{c}\sin\alpha$. The numerical aperture should be as large as possible for two different reasons :

i) the spatial resolution improves for larger *NA*

ii) the collection efficiency, i.e. the brightness of the image, increases very quickly with *NA*, quadratically for small apertures. The fraction of light collected for an isotropic light source is :

$$\frac{\Omega}{4\pi} = \frac{1}{2}(1-\cos\alpha) = \frac{1}{2}\left(1-\sqrt{1-(NA/n)^2}\right).$$

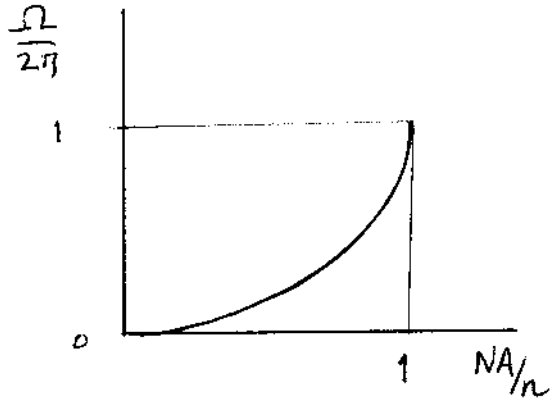

Figure 2.2 : Variation of the collection solid angle with numerical aperture NA. The increase is quadratic for low NA, and becomes even steeper for larger NA's.

Therefore, the main difficulty in manufacturing microscope objectives is to achieve a good correction of all aberrations (spherical, chromatic) also for off-axis rays with angles which can be larger than 60°. This is achieved by assembling a large number of lenses (sometimes more than 10), which have to be anti-reflection-coated for a good luminosity. Good microscope objectives are therefore quite advanced and expensive pieces of technology. Immersion oil objectives reach a NA of 1.4, corresponding to collection angles of 70° or more.

An ideal microscope lens will therefore image a point source as an Airy pattern, if a circular iris or diaphragm limits the aperture. The point-spread function (PSF) has therefore the classical form of the diffraction spot from a round hole. If the light collected is a Gaussian beam instead, the PSF is a Gaussian spot.

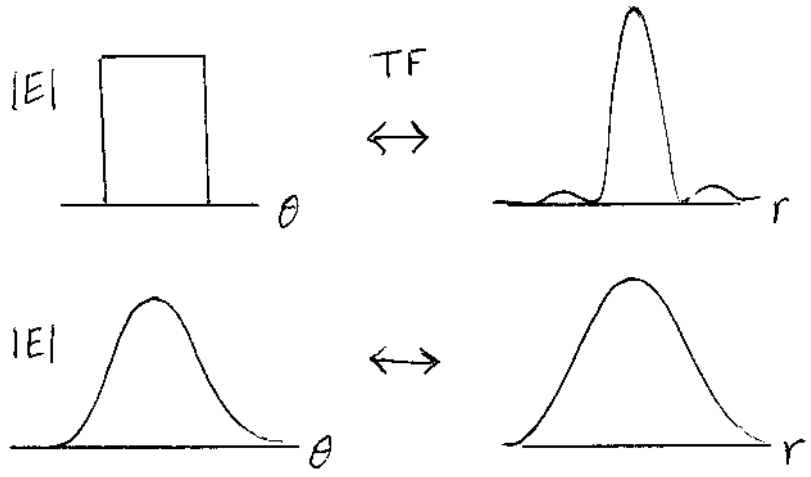

Figure 2.3: Angular distribution of the field in the lens aperture and Fourier transform intensity distribution in the image plane (point-spread function)

In the axial direction, the size of the PSF is approximately : $\frac{2n\lambda}{NA^2}$, and is known as the depth-of-focus or Rayleigh length. The 3D appearance of the PSF is thus an elongated (prolate, or cigar-shaped) ellipsoid. Decreasing this length is the third reason why the numerical aperture should be as high as possible. A shorter Rayleigh length means a stronger rejection of out-of-focus sources of background.

The numerical aperture is inversely proportional to the object refraction index. Therefore, it is of advantage to collect light through glass, or through high-index oil, whenever possible. Special water-immersion objectives are used for biological samples. To fully benefit from high index, the index must of course be matched between sample and objective glass. This is achieved thanks to immersion oil. A further advantage of immersion is the higher efficiency of fluorescence collection. An air gap leads to light losses by total internal reflection at the interface from high- to low-index media.

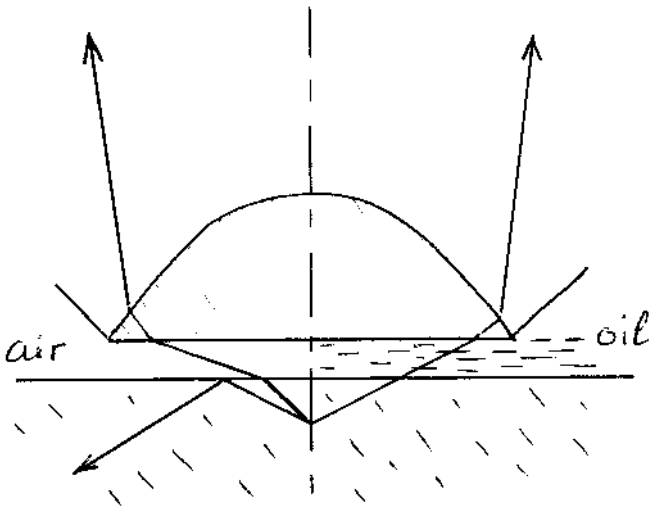

Figure 2.4 : Importance of immersion to collect light from an emitter in a high-index medium. In the case of an air gap, much light is lost by total internal reflection at the air-sample interface.

For emitters placed *at* an air-glass interface, the situation is even worse, as a large fraction of the emitted light (up to 90%) is sent into the high-index material. Collecting emission on the air side therefore entails a big loss of intensity.

2.2. Correction of aberrations

The ideal microscope objective would image a planar object onto a plane field (aplanetism), without distortion, and without change in image with wavelength (achromatism). To achieve this, the following aberrations must be corrected :
i) chromatic aberrations from the dispersion of glasses (their refractive index is larger for blue than for red light). Even with well-corrected objectives, the focus often moves by some microns when the wavelength varies over the visible spectrum.
ii) geometrical aberrations : spherical (change of focal point with distance from axis), coma, due to changes of the image point with ray direction, field curvature (image focussing is not obtained on a plane but on a curved surface), field distortion (pincushion or barrel images), etc.

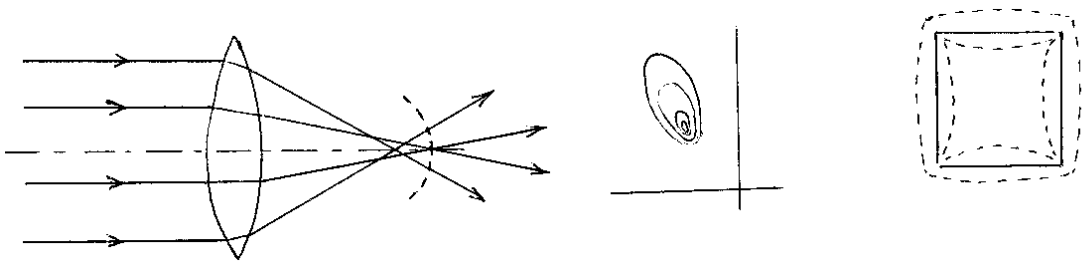

Figure 2.5 : Some geometrical aberrations : spherical aberration (left), leading to coma for off axis rays (middle). Focussing on a spherical field (left) instead of a planar one, image deformation as a pincushion or barrel (right)

Simple spherical lenses made out of ordinary dispersive glass suffer from all these aberrations and cannot fulfil the requirements of the ideal lens for large numerical apertures. For example, a plano-convex lens has spherical aberrations which are minimized by placing the convex surface on the side of the parallel beam. Aspheric singlets used in CD readers correct in principle perfectly for their focus, but they work only at one wavelength and have strong chromatic aberrations. To approximate the ideal lens' requirements, one uses combinations of spherical lenses possessing various radii of curvature, thicknesses, and materials, and one varies their positions. To design objectives and other multilens systems, special codes calculate imaging with rays far from paraxial for arbitrary systems of lenses. A good objective may contain as much as 10 lenses, which have to be positioned with specifications as narrow as microns for some of them. The air-glasses interfaces have to be anti-reflection coated to reduce

reflection losses, and small gaps between the lenses are bridged with a high-index medium, usually UV-polymerizable glue, after the respective position of the lenses has been adjusted by hand. Therefore, objective lenses are expensive and sensitive optical components.

Nowadays, most objectives are infinity-corrected. This means that they are not calculated to form their image directly in the intermediate image plane, but to form an image at infinity. Another lens called *tube lens*, then images the plane waves into the intermediate image. Some manufacturers use the tube lens to correct some aberrations. In that case, tube lens and objective must be used in combination for optimal correction. The advantages of infinity-corrected objectives are essential in confocal microscopy, polarization studies and spectroscopy, because plane waves are easier to filter and manipulate than spherical waves (for example they are not distorted by flat windows).

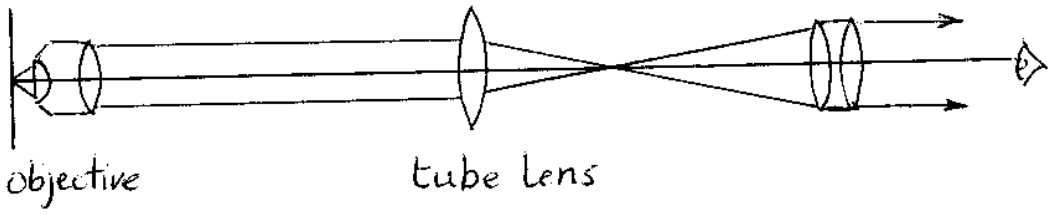

Figure 2.6 : Schematic principle of a microscope with infinity-corrected objective lens. The tube lens is used to obtain the intermediate image, which can then be seen with the eye piece.

2.3. Polarization structure at the focus

The electric field of a laser wave is a vector quantity, which gives rise to a complicated polarization structure in the case of high numerical aperture. We will briefly discuss the polarization of the field at the focus of a linearly polarized laser wave. For low NA, the polarization of the spot is the same as that of the incident beam. At the focal point itself, by symmetry, the polarization is also the same. At high NA, however, and for parts of the PSF away from the focus, interference of the incoming rays leads to deviations from this polarization. For large incidence angle and a linearly polarized incident beam, a simple drawing shows that the longitudinal (i.e., axial) component of the field presents two (weak) lobes in the focal plane, with a node in the center. The third transverse component (perpendicular to the axis and to the incoming polarization) is even weaker and presents four lobes. By using annular

illumination, and/or by introducing phase masks in the incoming beam, the polarization of exciting laser light at the focus can be manipulated, and can even present a PSF with a single lobe for the longitudinal polarization. This is of great interest to determine the 3D orientation of single absorbers, since the transverse polarization can be probed with linearly polarized light and normal illumination.

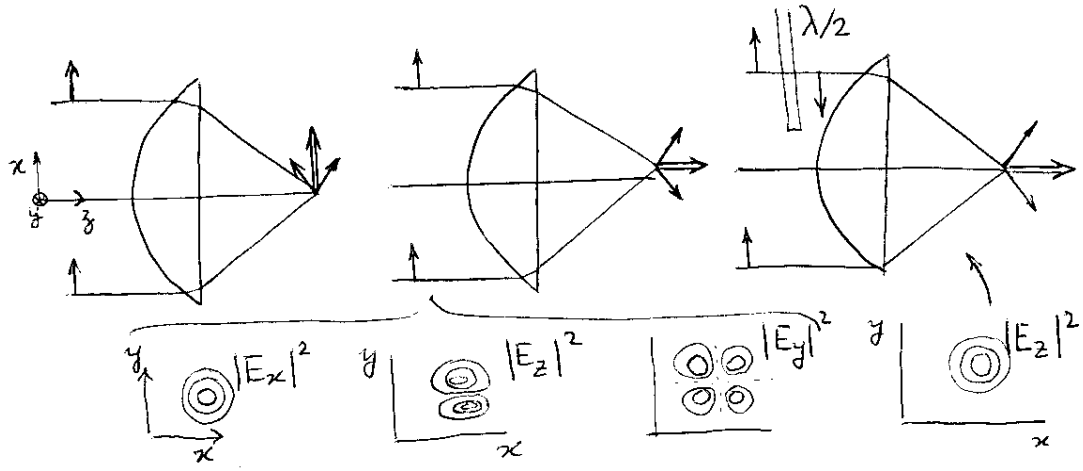

Figure 2.7 : Polarization of light focused from a linearly polarized beam. In the center of the PSF, the polarization is conserved (left). Away from the center, optical path differences lead to a longitudinal component of the field (middle). By introducing a retardation plate on part of the rays, the polarization can be manipulated, for example to obtain a strong longitudinal component with a single lobe. The intensity distributions are schematically represented in the lower part of the figure.

A similar, but distinct problem is to find the polarization structure of a wave radiated by a linear dipole at the focus, and collimated into a plane wave by an objective with large numerical aperture. In the case of a dipole lying in the focal plane, it can be shown (Fourkas Opt. Lett. 2001) that the polarization is linear throughout the field, parallel to the dipole along the horizontal and vertical directions (N, S, E, W), and significantly tilted in the NW, NE, SW, SE positions. In the case of a dipole perpendicular to the focal plane, the polarization is radial.

2.4. Various microscopy methods :

There are several ways to record images with a microscope. We briefly mention the most important ones for single-molecule studies:
i) confocal microscopy : in this method, only one point of the sample is imaged onto a photodetector. If the sample is moved in 3 dimensions, a 3D image is recorded. To reduce background, i.e. to insure that the signal arises only from the focus, a diaphragm or pinhole is inserted in one of the image planes. To scan the area to be imaged, one can move either the sample itself with piezo-electric transducers (sample scanning), or the focus by means of tilting mirrors (beam scanning).

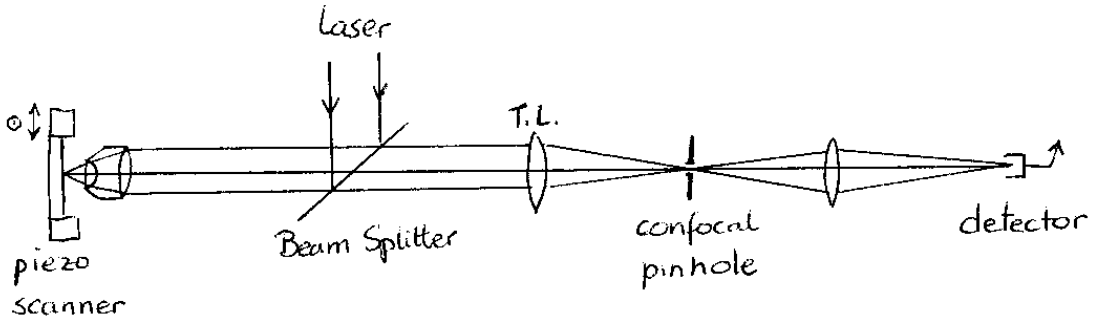

Figure 2.8 : Confocal microscope with sample scanning. The signal intensity is recorded as a function of the position of the sample.

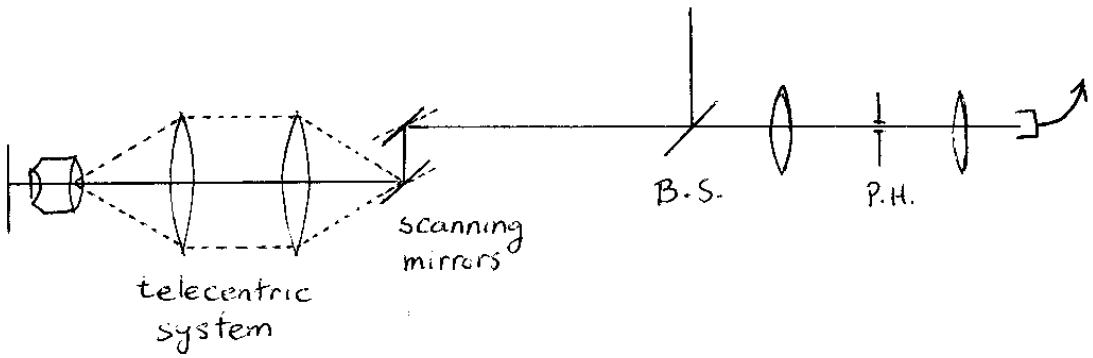

Figure 2.9 : Confocal microscope with mirror scanning . The scanning mirrors deflect the illumination beam in two orthogonal directions. The telecentric system images the laser spot on the mirror (or mirrors, in which case these mirrors have to be as close to each other as possible) on the entrance lens of the objective. The backwards traveling detection beam of course is refolded by the mirror(s) precisely onto the incoming path.

ii) wide-field imaging : in this method, a large part of the field is illuminated by an unfocused beam (epi-illumination), and the image is formed on a multi-channel detector such as a CCD camera or an image intensifier. In that case background arises from emissions below and above the imaged plane of the sample. This background can be significantly reduced by imaging Ronchi rulings in the focal plane and subsequent composition of two or three images. Such rulings are also used in structured-illumination microscopy to improve the resolution by a factor of up to two.
iii) To reduce the background, the excitation light can be sent at a large incidence angle on the surface, achieving total internal reflection (TIR). Fluorescence and other emissions can be collected either on the other side of the interface, or on the same side as the illumination. In that case, an immersion objective with large N.A. is of course necessary.

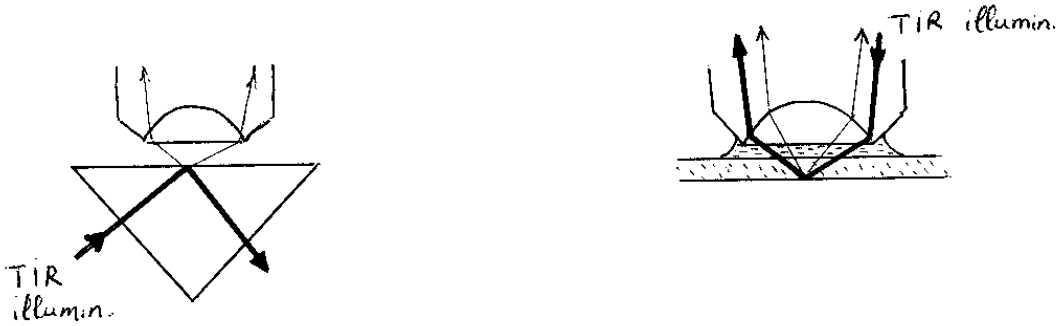

Figure 2.10 : Illumination of a sample by total internal reflection, either with a high-index prism in the case of a low index sample (left), or via the objective lens itself in the case of a sample close to an interface to a lower index (air or water, right).

Confocal microscopy is particularly useful to detect single molecules. The design has the advantage that only the focus is excited with high efficiency, and that fluorescence arising from other points does not reach the detector. The spatial selection is therefore performed in two steps, with equivalent performance:
- excitation selection, by focusing the laser beam on a small spot,
- detection selection, by detecting from the same area only.
Several other optical designs have been developed in the last twenty years with various elements for excitation and collection : single mode optical fiber, parabolic mirror, aspheric lenses, gradient index lenses, etc.

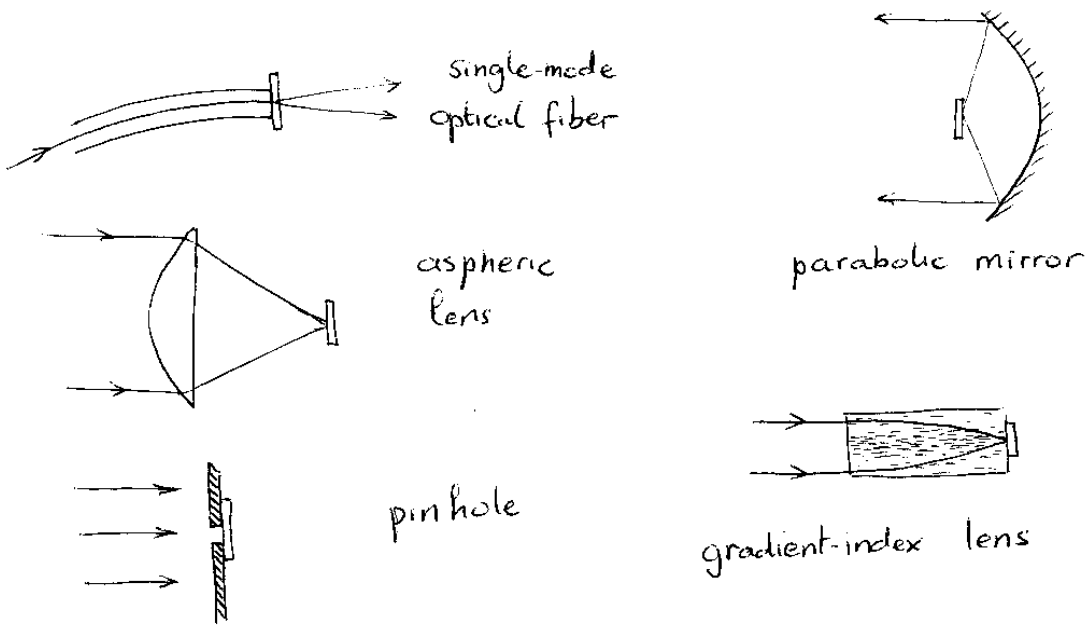

Figure 2.11 : Various elements used in single-molecule optics, besides microscope objectives.

2.5. Near-field optics :

To improve the resolution of optical microscopic images, and to reduce the selected volume in single-molecule studies, it is of great advantage to reduce the spot size below the Abbe diffraction limit. Optics at ranges smaller than the wavelength are called near-field optics. To produce and analyze optical fields with variations less than λ, interaction with microscopic objects is necessary. Near-field optics thus has to use small objects, usually tips or small apertures, to enhance or constrain the optical field. It has therefore much in common with scanning probe microscopies (STM and AFM): the scanning procedure, the importance of the tip, the stability requirements; However, the slow spatial variations of the optical field make it much more difficult to interpret and model the images obtained.

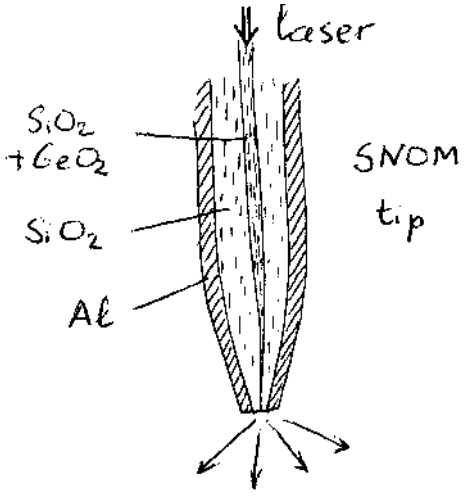

Figure 2.12 : Structure of a currently used SNOM tip. Incident light propagates in the core of a single-mode fiber, and reaches the tip. A small part of it is guided to the end and produces an evanescent wave around the aperture. Part of the light is also radiated in the far field across the aperture.

A common way to confine the optical field is to stretch an optical fiber, so as to obtain a conical tip, and to coat it with a thin, but opaque metal layer, usually aluminum. The end of the fiber is uncoated, and is therefore a small pinhole through which an evanescent light wave can pass. Alternatively, the end of the fiber can be cut with an ion milling machine (FIB). The diameter of the hole is often 50-100 nm. The transmission decreases very rapidly with diameter, approximately like the 6^{th} power for small sizes. For a 20 nm diameter, the transmission does not exceed 10^{-6}. A scanning near-field optical microscope (SNOM) can be used in excitation (via the fiber) or detection (or pick-up) mode. To detect fluorescent single molecules, it is important not to irradiate the sample too long, therefore the excitation mode is preferable. The tip is scanned across the sample, and the total fluorescence is collected by an auxiliary optics (a microscope objective) as a function of tip position. While scanning, the distance between tip and surface must be kept constant. Several methods can be used, among which shear-force AFM is very common. To detect the weak force from the substrate, the tip is glued to a tuning fork. The presence of the surface manifests itself by a shift and broadening of the fork's resonance. The phase of the oscillation can also be used as error signal for the stabilization of the tip's altitude.

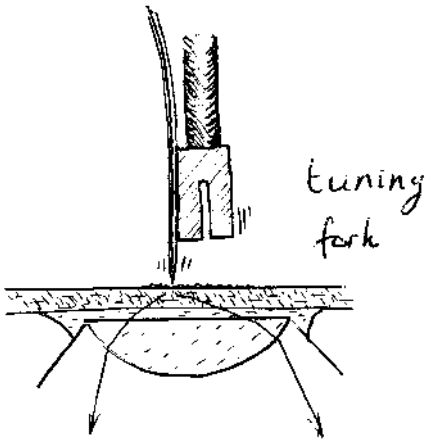

Figure 2.13 : Optical path in a SNOM where the tip is used for excitation. Detection is done in far-field, by means of a microscope objective. The distance of the tip to the sample is regulated by atomic-force microscopy, detecting the change in oscillation of the tuning fork carrying the tip.

A SNOM gives in principles access to spatial resolutions less than 100 nm, as was demonstrated by van Hulst's group; However, the operation of a SNOM is much more demanding than that of a far-field confocal microscope, and in many cases,

particulary in single-molecule studies, the increase in resolution does not justify the additional work. Moreover, low temperatures are even more difficult, and fluorescent objects below the surface cannot be accessed in near-field.
A recent development of near-field optics is plasmonics, in which resonances of the metal structures for surface plasmon resonances give rise to strongly enhanced and localized fields. These plasmonic effects will be briefly described in the last chapters in the case of metal nanoparticles.

Exercise 2.1: Use the Gaussian beam formulas below to find the above-mentioned depth of focus (also called Rayleigh length $L = \pi \frac{{w_0}^2}{\lambda}$*) in the axial direction.*

$$E(x,y,z) = E_0 \frac{\gamma(z)}{\gamma_0} e^{ikz} \exp\left[-\gamma(z)(x^2+y^2)\right],$$

with $\frac{1}{\gamma(z)} = {w_0}^2 + 2i\frac{z}{k}$.

(express the beam waist w_0 *as a function of the half-aperture angle* α*, and use the paraxial approximation* $\tan\alpha \approx \sin\alpha$*).*