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 (usually 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.

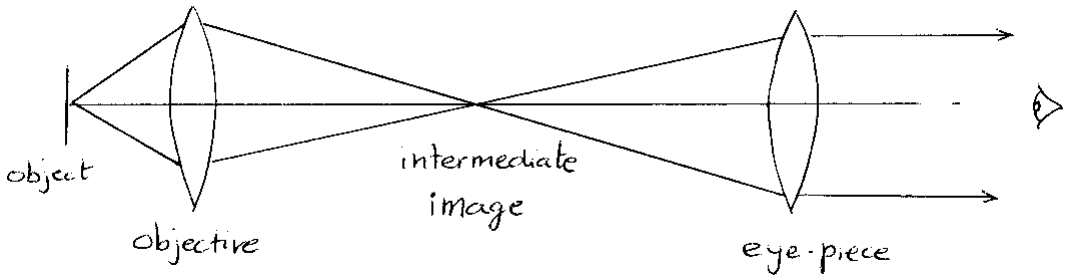

Figure 2.1 : Principle scheme of an optical microscope. The objective lens has a much shorter focal length than the eye-piece, in order to magnify the intermediate image (usually by a factor 40-100).

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. Applying Snel's law, you can check that the numerical aperture is a conserved quantity when light crosses a flat interface. The above relation, due to Abbe, is equivalent to a Fourier (or Heisenberg) relation applied to transverse spatial coordinate and transverse wavevector, $K = n\frac{\omega}{c}\sin\alpha$.

The numerical aperture of a good microscope objective should be as large as possible for two different reasons :

i) the spatial resolution improves in all three dimensions 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 :

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

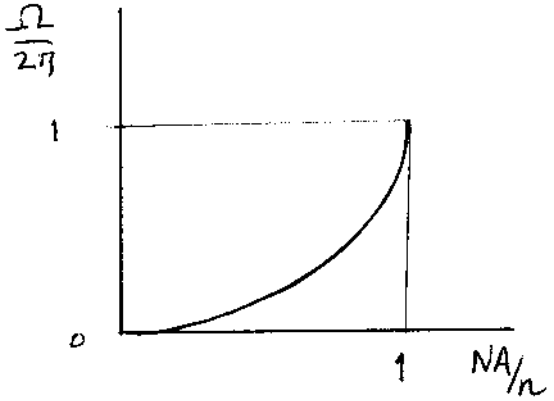

Figure 2.2 : Variation of the collection solid angle with numerical aperture NA. The increase is quadratic for low NA, and becomes even steeper for larger NA's.

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 high image brightness. 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. Let us discuss in more detail the three-dimensional shape of the image of an ideal laser beam, or conversely of an ideal point source.

Transverse point-spread function:

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) at the focus of the microscope can be controlled by adapting the diameter of the incident beam to the back-aperture of the objective. If the incident laser beam, assumed to be Gaussian, is much larger than the aperture, the transmitted wave front has the classical shape of the diffraction spot of a plane wave diffracted by a sharp round hole. This case is called *overfilling*, and leads to an Airy diffraction pattern. The ideal Airy pattern has 86% of the transmitted intensity in the central spot (Airy disk) and 7% of the intensity in the first ring. If the incident laser beam entirely fits inside the aperture

instead, the PSF is a Gaussian spot as Fourier transform of the Gaussian laser beam. In this case, called *underfilling*, the spatial resolution is worse but the light spots are more concentrated, without rings, usually leading to clearer images of crowded samples.

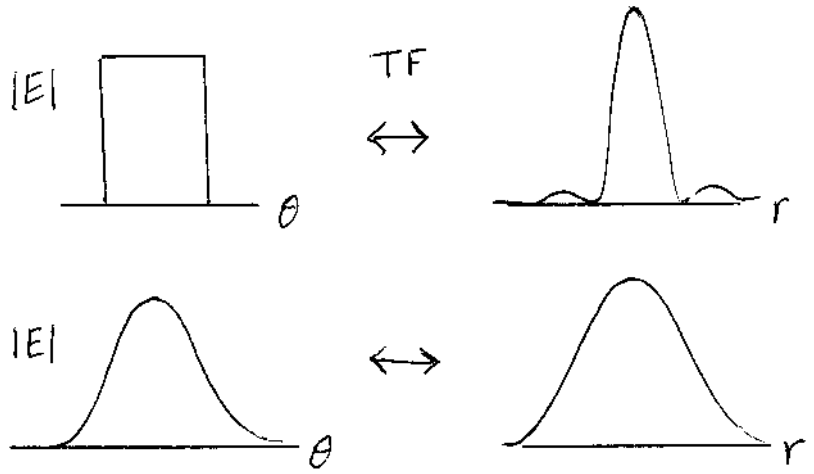

Figure 2.3: Angular distribution of the field in the lens aperture and Fourier transform intensity distribution in the image plane (point-spread function).

Axial 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, cigar-shaped) ellipsoid. Decreasing this length and focussing better along the axial direction is an additional reason to make the numerical aperture as high as possible. A shorter Rayleigh length means a better rejection of out-of-focus sources of background, and reduced illumination of out-of-focus sample parts. From the above discussion, we deduce that the volume of the point-spread function scales as the inverse *fourth power* of the numerical aperture. The background in single-molecule experiments scales in the same way.

The numerical aperture is proportional to the object's refractive 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 front lens. 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, as illustrated in Fig. 2.4.

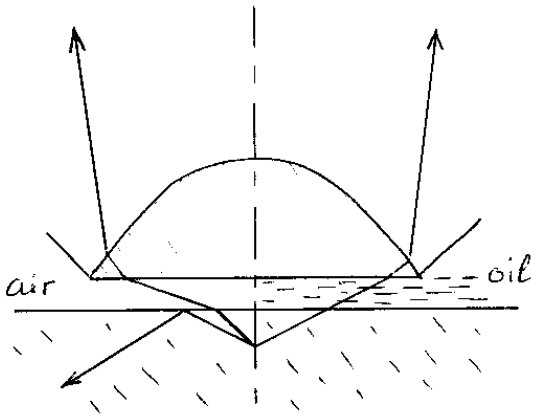

Figure 2.4 : Importance of immersion to collect light from an emitter in a high-index medium (right). In the case of an air gap (left), much light is lost by total internal reflection at the sample-air interface.

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 "sucked" into the high-index material. Collecting emission from 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 (aplanatism), without distortion, and without change in image with wavelength (achromatism). To achieve this, the following aberrations must be corrected (see the five Seidel aberrations http://www.quadibloc.com/science/opt0505.htm):

i) chromatic aberrations from the dispersion of glasses (their refractive index is larger for blue than for red light, but by different amounts for different glasses). Even with well-corrected objectives, the focus often moves by some micrometers when the wavelength varies over the visible spectrum.

ii) geometrical aberrations : spherical aberrations (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.

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-glass 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 an UV-polymerizable glue, after the respective position of the lenses has been adjusted by hand. Therefore, objective lenses are expensive and sensitive optical components.

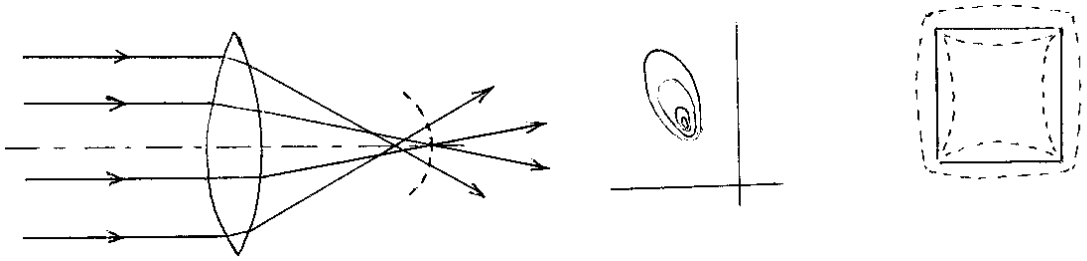

Figure 2.5 : Some geometrical aberrations : spherical aberration (left), leading to coma for off axis rays (middle). Focussing on a spherical field (left) instead of a planar one, image deformation as a pincushion or barrel (right)

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 those plane waves into the image, usually formed on a multichannel detector such as a charge-coupled device (CCD). Some manufacturers use the tube lens to correct some aberrations of the objective lens. 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).

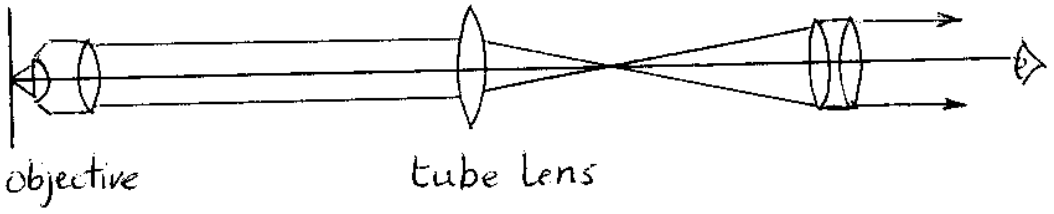

Figure 2.6 : Schematic principle of a microscope with infinity-corrected objective lens. The tube lens is used to obtain the intermediate image, which can be formed onto the sensitive area of a multipixel camera, or seen with the eye piece.

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 at the focus in the case of high numerical aperture. We will briefly discuss the polarization of the field at the focus when a linearly polarized laser wave is sent into the objective. For low NA, the polarization of the spot is the same as that of the incident beam, and a scalar description of the field is sufficient. For a high NA, the polarization at the center of the focal spot is conserved for symmetry reasons. However, as we look at regions of the PSF further away from the center, interference of the fields carried by the different incoming rays leads to significant deviations from the incident polarization, say x (see Fig. 2.7). For large incidence angle and a linearly polarized incident beam, a simple drawing shows that the longitudinal (i.e., axial along z in Fig. 2.7) component of the field presents two (weak) lobes in the focal plane, with a node line passing through the center and perpendicular to the incident polarization. The third component, y, of the polarization is also transverse, perpendicular to the axis and to the incident polarization. The intensity of this polarization component is even weaker than the axial one, and presents four lobes, in the quadrants defined by the incident polarization and its perpendicular. The polarization of light at the focus can be further manipulated by using annular illumination, and/or by introducing phase masks in the incoming beam. With a suitable phase mask, it is possible for example to create a PSF with a single lobe for the axial polarization. This is of great interest to determine the 3D orientation of single absorbers, since an axial polarization measurements complements standard in-plane measurements with linearly polarized light.

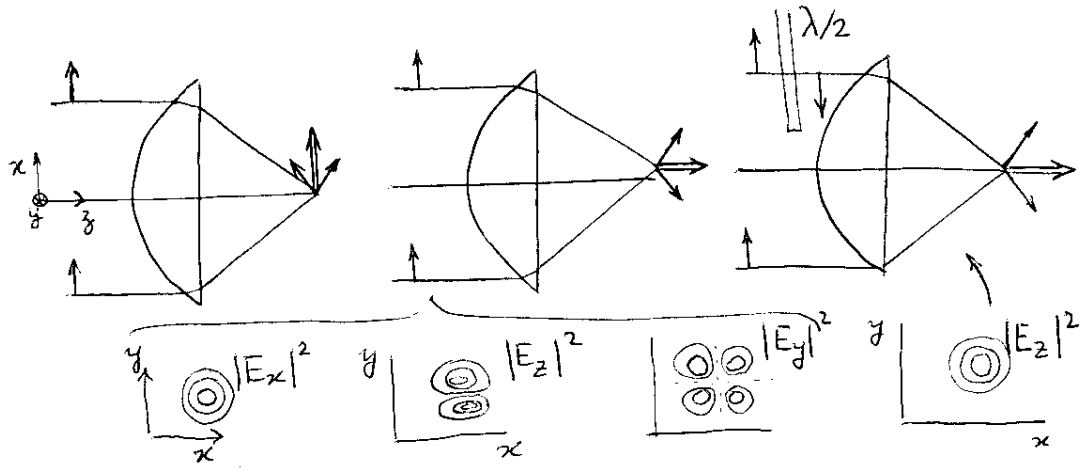

Figure 2.7 : Polarization of light at the focus of a linearly polarized beam. In the center of the PSF, the polarization is conserved (left), as all light paths reach the focus with exactly the same phase. Away from the center, optical path differences and vector interference lead to longitudinal and perpendicular transverse components of the field (middle). By introducing retardation plates on parts of the beam (right), 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 further effect of the large numerical aperture is a slight elongation of the PSF along the direction of the polarization for a linear incident polarization. For vertical incident polarization, let us define the two rays coming from the top and bottom of the objective as North (N) and South (S) directions, and those coming from the horizontal extreme points as East (E) and West (W). The field components coming from the E and W directions are fully projected at the focus, whereas the N and S components are projected with the cosine of the rays' angle with the optical axis. Therefore, as the vertical direction has a narrower angle distribution than the horizontal one, the corresponding PSF is broader in the vertical direction than in the horizontal one. This effect does not exceed 20% for usual values of the NA.

The polarization structure of the wave radiated by a linear dipole placed at the focus of the objective and collimated by a high-NA objective lens is a related, but distinct problem from that of focusing a plane linearly polarized wave. 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), but it is significantly tilted in the NW, NE, SW,

SE positions. In the case of a dipole perpendicular to the focal plane, the polarization is obviously radial at all points of the field with a node at the center. For arbitrary dipole orientations, the polarization pattern is more complex and includes a node at the point corresponding to the dipole's direction, as a dipole doesn't radiate along its axis.

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:

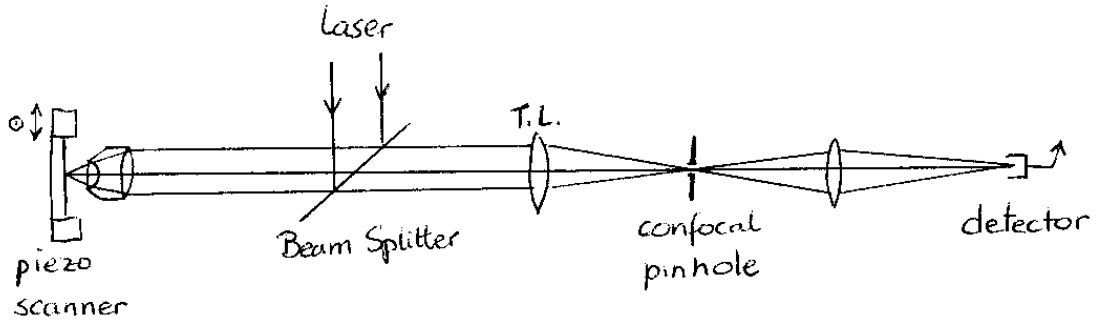

Figure 2.8 : Confocal microscope with sample scanning. The signal intensity is recorded as a function of the position of the sample.

i) confocal microscopy : in this method, only one point of the sample is imaged onto a single-channel 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. 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, each one with equivalent performance:
- excitation selection, by focusing the laser beam on a small spot,
- detection selection, by detecting from the same area only.
This spatial filtering improves the resolution slightly (the PSF enters in the illumination distribution and in the filtering efficiency), at the cost of a somewhat lower signal. More importantly, because of the faster decay of the total PSF in the axial direction, we obtain the important property of *optical sectioning*: only the slice of sample in focus within the depth of focus contributes to the signal. Without the

pinhole, all sample slices would contribute equally to the image because they receive the same illumination, producing a high back
ground.

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).

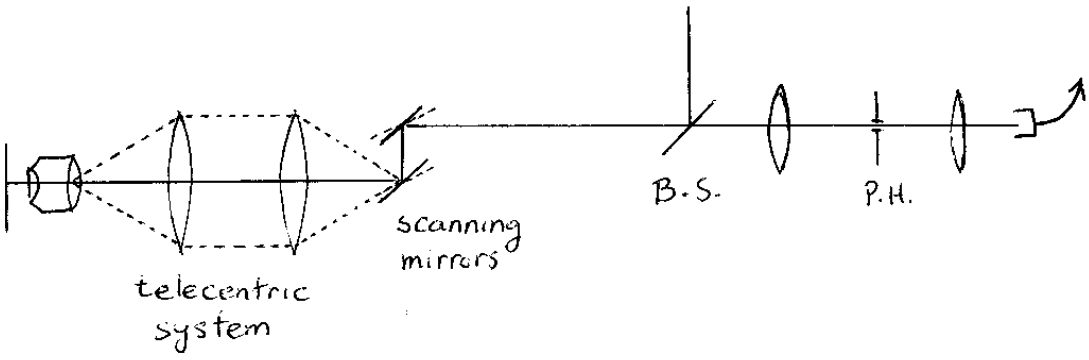

Figure 2.9 : Confocal microscope with mirror scanning . The scanning mirrors deflect the illumination beam in two orthogonal directions. The telecentric system images the laser spot on the mirror (or the two mirrors, in which case these mirrors have to be as close to each other as possible) on the entrance 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).

iv) light sheet illumination: to reject the signal from out-of-focus molecules and to reduce photobleaching in 3D samples, particularly cells and tissues, modern light sheet microscopes illuminate the sample sideways with a thin sheet of light. This requires special objectives with long frontal distances so that illumination and collection objectives, which are perpendicular to one another, can both approach the specimen sufficiently.

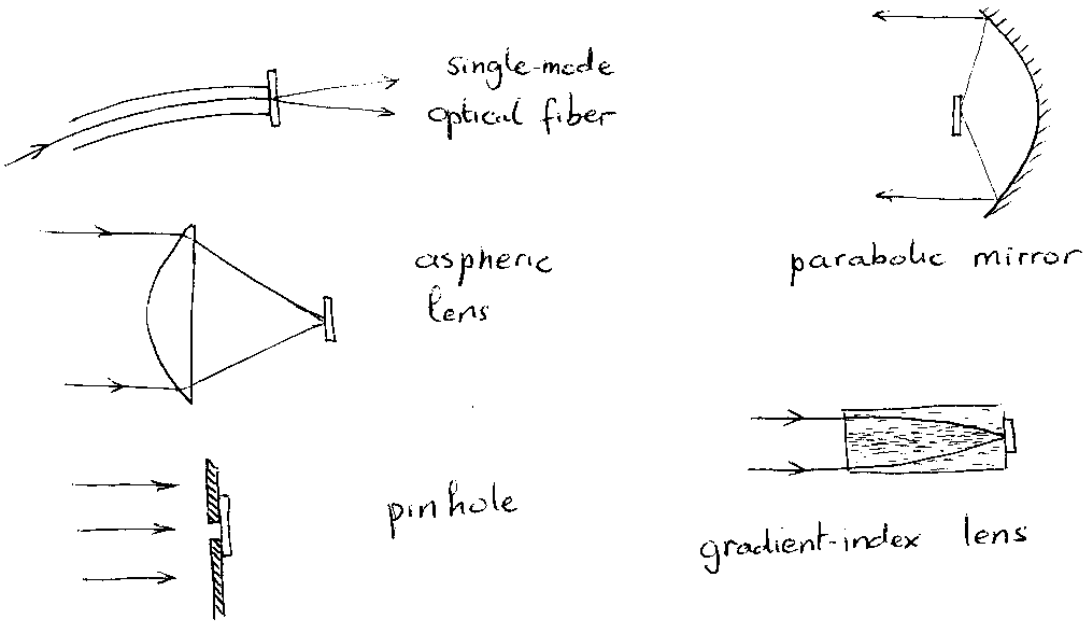

Figure 2.11 : Various elements used in single-molecule optics, besides microscope objectives.

Several other optical designs have been developed in the last thirty years for single-molecule spatial selection. They make use of various optical elements for excitation

and collection : single mode optical fiber, parabolic mirror, aspheric lenses, gradient index lenses, etc.

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 on distances much smaller than λ, interaction with microscopic objects is necessary. Near-field optics thus has to use small objects, usually tips or small apertures, to enhance and confine the optical field. Near-field optics therefore have 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.

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 auxiliary optics (e.g., 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.

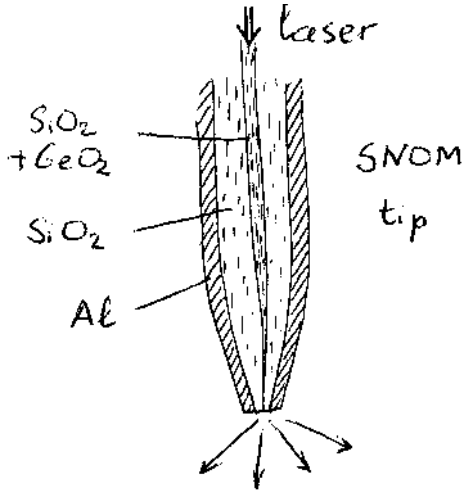

Figure 2.12 : Structure of a currently used SNOM tip. Incident light propagates in the core of a single-mode fiber, and reaches the tip. A small part of it is guided to the end and produces an evanescent wave around the aperture. Part of the light is also radiated across the aperture into the far field.

A SNOM gives 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, particularly in single-molecule studies, a modest increase in resolution does not justify the additional work. Moreover, near-field optics at 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 of this course in the case of metal nanoparticles.

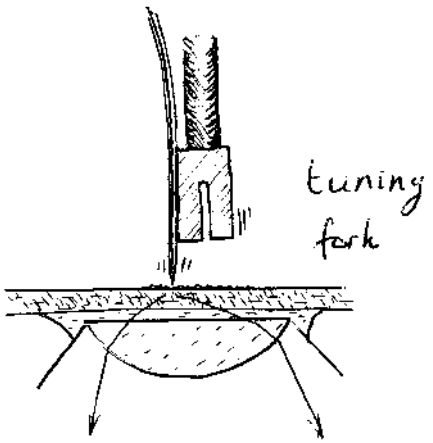

Figure 2.13 : Optical path in a SNOM where the tip is used for excitation. Detection is done in far-field, by means of a microscope objective. The distance of the tip to the sample is regulated by atomic-force microscopy, detecting the change in oscillation of the tuning fork carrying the tip.

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* $\tan\alpha \approx \sin\alpha$*).*

Exercise 2.2: Correction of chromatic aberrations. Consider the focusing of two beams with different wavelengths λ_1 *and* λ_2*, for which the focal lengths of the objective* F_1 *and* F_2 *are slightly different. Before reaching the objective, the second beam passes a telescope (two-lens afocal lens system) with magnification 1 and focal length* f*, independent of* λ*. By how much should the position of the second lens of this telescope be shifted to overlap the foci of the two wavelengths?*

Exercise 2.3: A polarization mask to control light polarization at the focus of a microscope is made of four quadrants with half-wave plates having the following slow- axis directions:
i) North: vertical
ii) South: horizontal
iii) East: 45° tilted in the NE-to-SW direction
iv) West: -45° tilted in the NW-to-SE direction
Discuss the polarization state at the focus for a vertical linear polarization of the incident beam? What about a horizontal linear polarization?
(Hint: a half-wave plate rotates linear polarization by twice the angle between polarization and slow axis).