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Optical Microscopy With Subwavelength Resolution

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Abstract

Optical microscopy is a valuable tool especially in biology and medicine as well as in chemistry and physics to determine various optical properties of materials. Unfortunately its resolving power has physical limitations. The resolution can not be much better than $\lambda/2$, which means around 200 nm in visible light. However, using clever tricks this barrier can be broken. For example, the position of fluorescent molecules can be measured with a nanometer accuracy. By selectively turning on just one at the time, it is possible to reconstruct the whole image. Another method is to use an excitation beam and a donut shaped depletion beam at the same time. With the intensity of the second beam getting higher, the nondepleted volume can get as small as we want. An obvious way of getting better resolution is also to use near field. In this case the light has to be spatially confined either by a small aperture or enhanced at a just specific point. Tapered fibers and sharp metal probes are used for this purpose. The probe is scanned near the surface of the sample pixel per pixel creating the whole image. Finally, also very specific material properties like Raman spectra can be measured using near field microscopy.

Contents

1 Far Field Microscopy

When we talk about optical microscopy we usually refer to a normal far field microscope that can be found in almost any laboratory. It is composed of a light source, an objective lens and an ocular lens or a camera. It is well known that the resolution of a optical microscope is limited by the wavelength of the light. However in the recent years a number of techniques based on normal far field microscopes have been developed, that overcome this limit delivering much better resolution.

1.1 Spatial Resolution

Spatial resolution is a measure of the ability to distinguish two separated point objects from a single object. The resolution is limited by the wavelength of the light used, but also depends on other factors. Usually it can not be significantly better than $\lambda/2$.

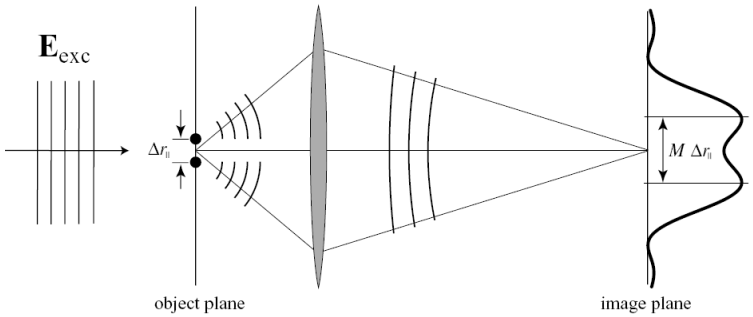

Figure 1: Experiment configuration: two point sources are imaged through a lens onto a screen. [1]

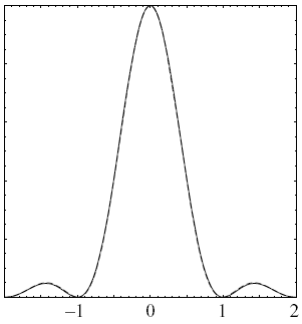

Figure 2: Point spread function for a single point source. [1]

To see how is the resolution limited by the wavelength we must first look at the point-spread function (PSF). The PSF determines the ability of an optical system to project a point light source to a imaging plane. The infinite size of the image is a direct consequence of frequency filtering. In the far field all frequencies higher than the frequency of the used light are lost. The spread function

for an ideal microscope is an airy disk, described by

$$I(\rho) \propto \left[\frac{J_1\left(\frac{2\pi \mathrm{NA}\rho}{M\lambda}\right)}{\frac{2\pi \mathrm{NA}\rho}{M\lambda}} \right]^2, \tag{1}$$

where ρ is the distance between the center and a point on the disk, NA is the numerical aperture, M is the magnification and λ is the wavelength. According to Abbe, the resolution is defined as the distance between two spread functions when the center of one PSF coincides with the first minimum of the second PSF. The distance between center and first minimum for a single PSF is calculated from (1) and is equal to

$$\Delta x = 0.6098 \frac{M\lambda}{\mathrm{NA}}. \tag{2}$$

The resolution limit is than

$$\min[d] = 0.6098 \frac{\lambda}{\mathrm{NA}}. \tag{3}$$

For example if we use a high numerical aperture oil immersion objective (NA=1.4) and we observe in blue light ($\lambda = 450$ nm), the resolution limit is about 200 nm.

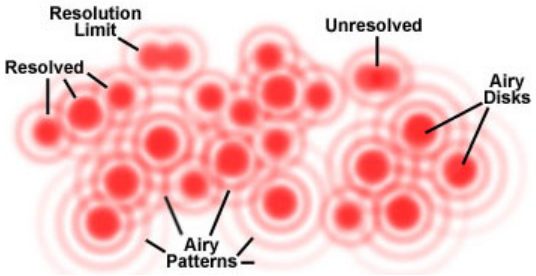

Figure 3: A number of point sources as seen under the microscope. Each source is visible as an Airy disk. If two point are too close to each other, we can not resolve them any more. [2]

However in a noiseless system we can in principle always decomvolve the two spread functions no matter how close they are to each other. If we know in advance some properties of the system we observe, we can achieve better resolution. Other far field field techniques can also achieve better resolution, as we will see in the following sections, so the above resolution criteria is not so strict.

1.2 Position Accuracy

In the previous section we discussed when we can distinguish two point sources close together. With a single source it is possible to determine its position with a much better accuracy than the resolution of the system used. The measurement of the position is not limited by the wavelength, but just by the quality of the captured data.

For example we observe a fluorescent molecule with an optical microscope and a CCD camera. On the CCD sensor the image of the molecule looks like an Airy disk (1). So we have a area of brighter pixels at the center where the image is located. To measure the position we must determine the center of this bright area of pixels. This can be done in a number of ways like calculating the

center of mass, or by fitting with the spread function. The position accuracy is mostly limited by the amount of noise on the CCD sensor and the number of photons emitted by the source, so how accurate is the measurement of a single pixel brightness. Position accuracy of couple of nanometers can be routinely achieved.

Furthermore, if two object emit photons that can be distinguished by for example polarization, wavelength or time of arrival, the two object can be distinguished, although they are closer together than the resolution limit.

1.3 Far Field Microscopy Techniques

1.3.1 PALM

For example, we want to observe a sample densely occupied by fluorescent molecules. In the Section 1.2 we saw that it is possible to achieve a position accuracy for a single molecule, which is much better than the resolution. All we need is to somehow lit a single molecule, determine its position and turn it off. Than lit another one and so on, to get the position of all molecules in the sample. However this is not so easy to achieve and only recently a technique that uses this principle has been demonstrated [3]. It is called photo-activated localization microscopy or shortly PALM.

PALM uses specially designed fluorophore molecules, that must be first activated by UV light in order to become fluorescent. The sample is infiltrated with this dark fluorophores. A weak flash of UV light is used to activate the molecules. Because photoactivation is random process, just a few, well separated molecules are activated. Now the these molecules are observed and their position is determined very accurately. Under the action of light, used to observe their fluorescence, the molecules photobleach. Than new fluorophores are activated by UV light and again observed until they photobleach. The process is repeated many times, building up an image molecule-by-molecule. Because the molecules were localized at different times, the "resolution" of the final image can be much higher than that limited by diffraction. The resolution achieved in this way is of order of couple nanometers [3]. By using interferometric methods [4] or optical astigmatism [5] also the vertical position of molecules can be measured to sub 20 nm resolution.

The PALM method is in principle very simple, because it uses standard equipment. You just need a microscope, appropriate light sources, filters, EMCCD camera and software. However specifically engineered fluorophores must be used, that can be photoactivated. The other mayor disadvantage of this technique is that it requires hours to take a single high resolution image. This limits the use of PALM just to static systems.

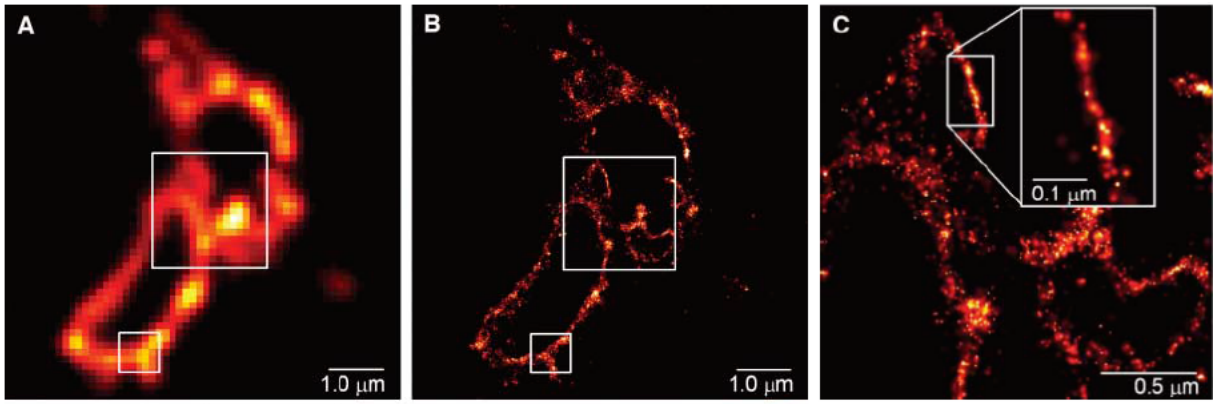

Figure 4: (a) Nonprocessed image of the sum of light coming from all molecules. (b) PALM image of the same region. (c) Higher magnification of the larger boxed region in (b). Molecules arranged in a line of the width 10 nm are clearly seen (inset). [3]

1.3.2 4Pi Confocal Microscopy

In a conventional microscopy we collect the light from whole thickness of the specimen. The parts of the sample that are out of focus are visible as blurred flares. In a confocal microscope a pinhole is used, that transfers only the light that comes from a specific point in the z direction. The pinhole is than scanned across the sample to get a picture of a slice of the specimen. If the focal point is scanned also in the z direction we can get a 3D image of the sample. The lateral resolution of confocal microscope is the same as for a conventional microscope ($\sim$ 200 nm), but the axial resolution is at best 500 nm to 800 nm. To achieve better axial resolution various techniques have been developed.

In a 4Pi confocal fluorescence microscope [6, 7] two opposing microscope objective lenses are used to illuminate a fluorescent object from both sides and also to collect the fluorescence emissions from both sides (Fig. 5a). Constructive interference of either the illumination wave fronts in the common focus or the detection wave fronts in the common detector pinhole result in an axial resolution four to seven times higher than that in a conventional confocal fluorescence microscope. Unfortunately, in this way two satellite interference spots are produced above and bellow the main focus (Fig. 5b). These spots produce two axially shifted ghost images of the recorded structure. However the images can be decomvoluted and filtered out to produce a correct picture.

Since the first 4Pi microscope in 1992 [6], this technique has largely evolved and today you can buy a beam scanning two photon excitation 4Pi confocal microscope (Leica TCS 4PI) with axial resolution of 80 nm.

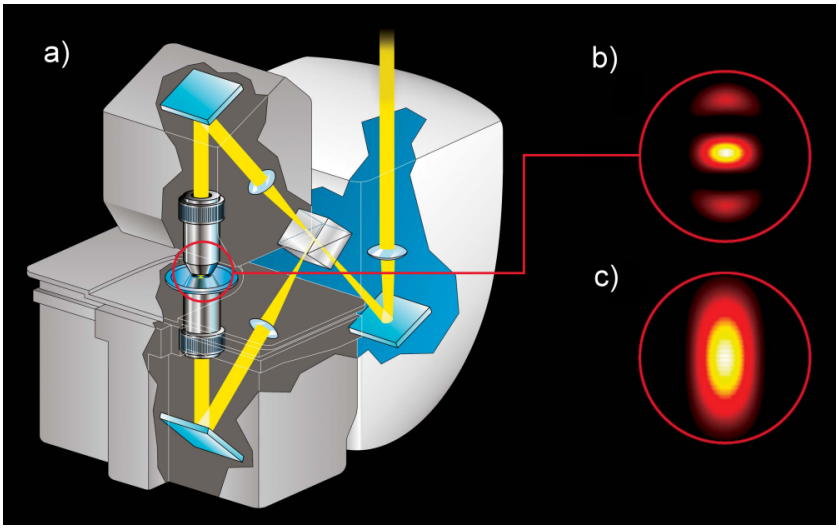

Figure 5: (a) A part of Leica 4Pi confocal microscope containing two opposing objectives. (b) Point spread function of a 4Pi microscope in the X-Z plane compared to (c) point spread function of a confocal microscope. [8]

1.3.3 STED Microscopy

Another very interesting approach to broke the diffraction barrier is the stimulated emission depletion (STED) microscopy [9, 10]. The technique consist of two beams of light that overlap in the focal point. The first beam is used to excite the flourescence molecules like in a conventional fluorescence microscope. The molecules than relax from this higher state to the ground state and emit fluorescent light. In the case of STED a second beam of light with different wavelength is used to actively bring the molecules back down to the ground level, but just in a specific region of the specimen. This depletion is done by stimulated emission, just like in a laser. The depletion beam is shaped like a doughnut and contains a node of zero intensity at the centre of the focus. So, just the molecules that are in the center of this beam are still in excited state and can irradiate fluorescent light that can be detected. This spot is than scanned trough the sample to get a high resolution 3D image.

Let's look in more detail what is happening at the focus and see why the resolution can be better than the diffraction limit. The intensity of the doughnut shaped depletion beam is decreasing continuously towards the center and is equal to zero only in the center itself. This is important because the stimulated emission takes place only if the intensity of the depletion beam is high enough. So, from the center outwards we reach a point where the intensity is high enough for the stimulated emission to take place. By increasing the intensity of the second beam we can arbitrary reduce the size of this non-depleted zone from which we than observe the fluorescence. In this way the resolution of microscope is no longer limited by the wavelength of the light, but is purely a question of technical design. Resolution as good as 25 nm has been reported [11].

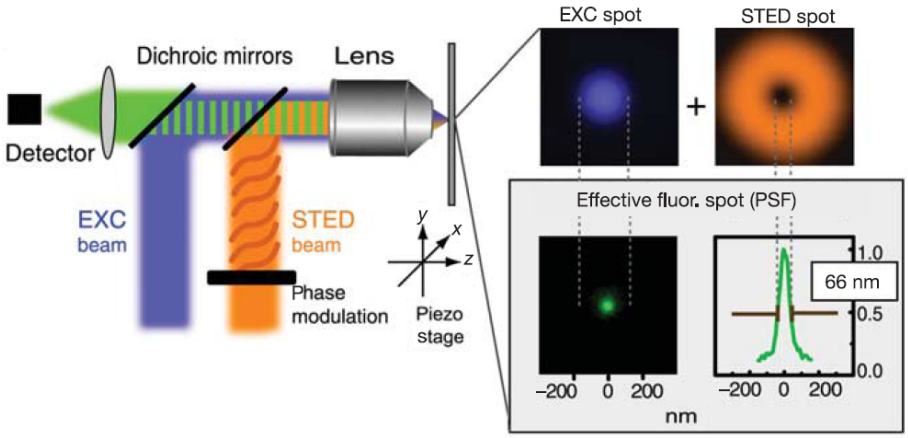

Figure 6: Principle of a STED microscope: excitation and doughnut shaped depletion beams are focused in the same spot, creating an effective flourescent spot that is much smaller than in a conventional microscope. [9]

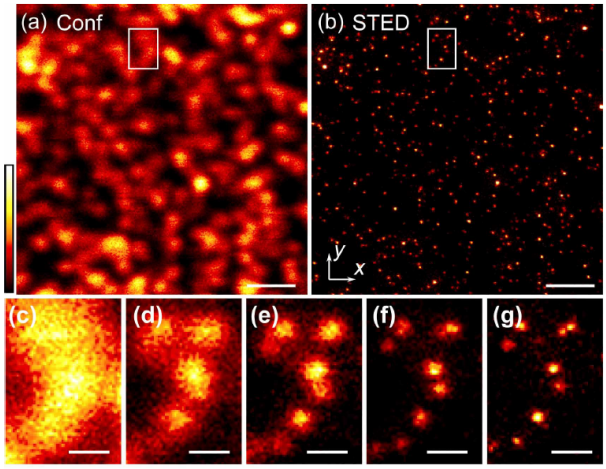

Figure 7: (a) Confocal and (b) STED image of 24 nm fluorescent beads on a cover slip. (c-g) The area of the white rectangle shown in (a) and (b) recorded with increasing STED intensities. The increase in resolution is clearly visible. Scale bar in (a,b) 1 μm, in (c-g) 200 nm. [11]

2 Near Field Microscopy

2.1 Principles

In previous sections we shown that using various tricks the diffraction limit can be beat, delivering images with amazing resolution. However techniques like PALM and STED only work with fluorescent molecules. But usually we want to observe nonfluorescent samples and measure their spectral properties, like for example Raman spectrum. In this case the only solution is to use near field microscopy.

In far field microscopy spatial frequencies associated with evanescent field are lost upon propagation from source to detector leading to diffraction limit. The idea of near field microscopy is to capture this near field components. Unfortunately, evanescent waves do not propagate and can not be captured by conventional optics. They are confined close to the surface of the object and decay exponentially away from the surface. They can be captured only if we bring an wave carrying object in the region within the first few tens of nanometers of the specimen surface. Such an light carrying object can bi either an illuminated sharp metal tip or a tiny aperture in a metal screen.

2.2 Near Field Microscopy Techniques

2.2.1 SNOM

The scanning near-field optical microscopy (SNOM) uses an optical waveguide with a nanoaperture at the and. The sample is either illuminated through this aperture or the light is collected through the aperture. The probe is than rastered across the surface in order to get the whole picture. The concept of SNOM was first published by Synge [12] in 1928. In 1984 Pohl et al. [13] and Lewis et al. [14] used this technology in practice.

To get subwavelength resolution the probe has to be very close to the surface, but not touching it. Distance must be also kept constant when the probe is rastered across the surface. The light itself is not enough to control the distance, since the optical properties of the surface are not known in advance and the amount of reflected/transmited light does not give the correct distance. A mechanical principle must be used instead. The optical probe is attached to a tuning fork, so that it vibrates laterally with an amplitude of couple of nanometers (Fig. 8a). The vibration amplitude is damped when the tip is close to the surface due to the interaction with the surface. The oscillation amplitude is than used as a feedback to maintain the tip at a constant distance from the surface. Another method is to use vertical oscillations as it is done in AFM taping mode (Fig. 8b). In this later case the tip and the sample can also be immersed in water enabling the observation of biological samples.

There are basically a number of SNOM modes of operation in respect to where the light collected and how the sample is illuminated. The probe can be used as a light source or can collect light or both. In the same way a far field microscope can be used as source of light or a detector, either in reflection or transmission through the sample. In Fig. 9 four most used arrangements are shown.

There are a number of material properties of a sample that can be characterized on a nanoscale by

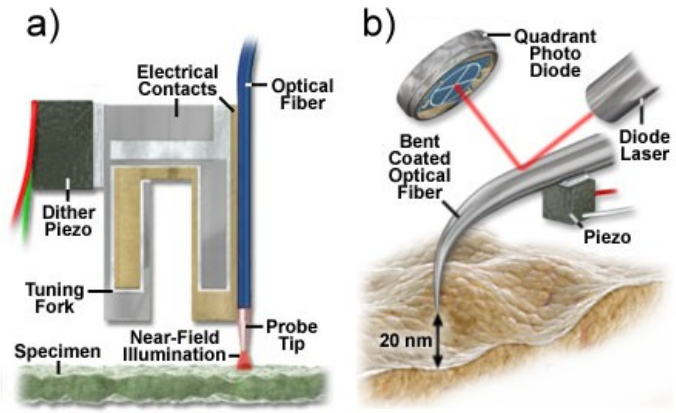

Figure 8: Tip-surface distance control: (a) shear-force feedback, (b) tapping-mode feedback. [15]

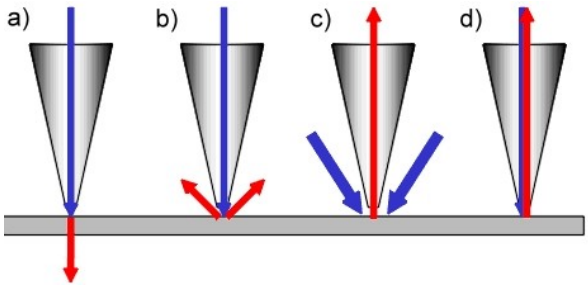

Figure 9: SNOM modes of operation: (a) transmission mode imaging, (b) reflection mode imaging, (c) collection mode imaging and (d) illumination/collection mode imaging. [16]

SNOM. The most common are index of refraction, reflectivity, transparency and birefringence. Also magnetic properties and mechanical stress can be measured if they influence optical characteristics of the material. Furthermore, fluorescence and Raman spectra can be acquired, even from single molecules. By using a feedback to control the distance between the probe and the surface also topographical or force data from the specimen are collected in the same manner as the atomic force microscope. In this way we get two separate data sets (optical and topographical) that can be compared to determine the correlation between the physical structures and the optical contrast.

Basically two types of probes are used for SNOM: uncoated tapered fibers and aperture probes. Tapering of optical fiber can be done either by etching or by heating and stretching the fiber until it brakes. The apex diameter for such tapered fibers is typically about 50-150 nm. To achieve higher resolution, the aperture at the apex must be smaller than that. For that reason the tapered fibers are coated with a metal layer and a small hole is made at the end of the tip. The hole can be formed by itself if the metal is deposited from such an angle, that it not covers the apex itself (Fig. 11a). Other methods include focused ion beam micromachining, mechanical punching the hole with a sharp tip and by using standard silicon micromachining techniques. A number of different SNOM probes can be purchased on the market.

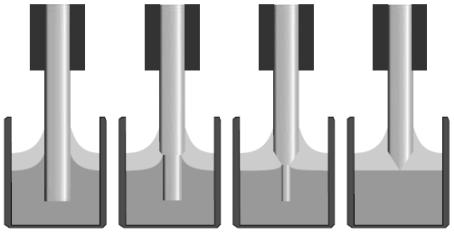

Figure 10: Etching of an optical fiber in a HF solution to achieve a sharp tip. [1]

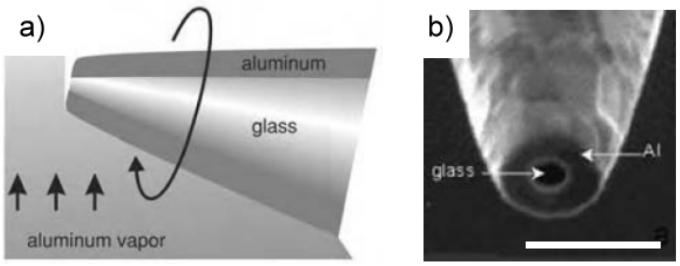

Figure 11: (a) Self-aligned formation of an aperture by metal deposition. The deposition takes place at an angle slightly from behind while the tip is being rotated. (b) SEM image of a aperture made in this way (scale bar is 300 nm). [1]

The best achieved resolution for SNOM is around 30 nm, limited by the probe manufacture. The effective diameter of an amperture can not be smaller that twice the skin depth of the metal used for coating the glass fibre. The skin depth is usually between 6 and 10 nm allowing the aperture to be around 20 nm in diameter. However, for such small apertures the transmission of light is very low. Typically, resolution around 100 nm is routinely achieved. However it is sometimes difficult to correctly interpret the captured image since artifacts can appear, as in any scanning probe microscopy. SNOM is also limited to surfaces of samples. PALM and STED are in this

respect a better tool, but they only work in florescence. On the other hand, SNOM can be used not only form imaging, but also for determination of other physical and chemical properties on a nano level.

2.2.2 ASNOM

Instead of a small aperture, a sharp tip can be used to confine light. The near field is created by any irradiated small material structure. Depending on the material properties, the field can be enhanced and than detected in far field. Apertureless near-field optical microscopy (ASNOM) uses this principle quite successfully. A sharp metal tip is brought close to the surface and illuminated by a tightly focused beam. The scattered light is than captured with a standard microscope objective. As a probe also a standard AFM tip can be used.

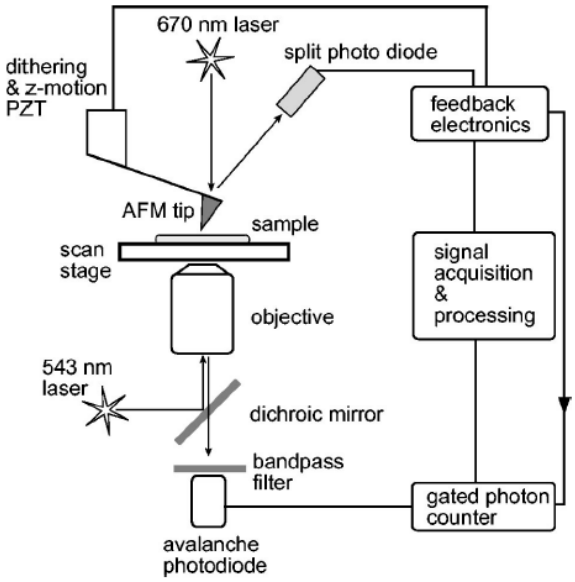

Figure 12: Principle of ASNOM operation. [1]

The light intensity near a tip is enhanced because it interacts with the metal and oscillates the electrons. If the metal is of a finite size, geometry dependent resonances can occur, called *plasmon resonances*. A major problem with field-enhancing structures is that they usually have to be excited using far-field illumination. The illumination from a diffraction-limited area around the tip also contributes to the final signal. The ratio between the areas associated with external excitation and near-field excitation is of the order 1000. This means that the enhancement under the tip must be higher than 1000 for the signal to be strong enough. However, if the nanoobjects that are the source of final signal have a very low surface coverage, or we have a single species in the illumination focus, than the background signal is not that important any more.

Similarly as SNOM, also ASNOM can measure different material properties, among others Raman and fluorescence spectra of single molecules can be captured. Sub 10 nm resolution has been achieved for fluorescence ASNOM [17].

3 Conclusion

With Abbe resolution limit as an fundamental obstacle, it seemed impossible to achieve better resolution with light microscopes. However in 1984 this barrier was for the first time broken [13, 14]. Since than a huge number of different near-field as well as far-field techniques have been developed that have the resolution much better than $\lambda/2$. The resolution is getting better virtually from month to month. Who knows, maybe in the future we will be able to see even atoms with optical microscopy.

Subwavelength optics is very interesting to semiconductor industry, since they want to make smaller and smaller integrated circuits. It has been shown that is possible to use SNOM to irradiate the photoresist achieving about 70 nm resolution [18]. However, this approach is not scalable to production, since the process is not parallel. Recently three techniques have been developed, that use far field illumination to achieve subwavelength resolution [19, 20, 21]. They use a principle very similar to STED. One beam activates the patterning chemistry while the other donutlike shaped beam suppresses the activation. Features as small as 40 nm have been created. It is possible that a similar concept will be used in the future to manufacture integrated circuits for our everyday electronic devices.

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