Microimaging

Imaging bees, polen and atoms

March 23, 2006, Thomas Wendler

Abstract

Microimaging refers to a group of imaging modalities that go beyond the limits of spatial resolution of the human eye into the microscopic world. The different image modalities within can be divided according to the nature of the signal measured, the dimensions imaged and the nature of the magnification. This review will deepen mostly in the two major modalities of magnification and try to make them clear to the reader through the explanation of the most important microimaging techniques.

1 A view into the invisible world

Microimaging is a widely used term for imaging the world of small things. The word itself is formed by the term "micro", forged in the ancient Greece meaning small [1] and the term "imaging", i.e. the process of producing images [2]. Microimaging is thus the process of making images of small entities.

The adjective "small", however, is rather subjective, but implicitly includes the dimensions of space: length, area or volume. In order to obtain an objective definition for microimaging a line at the limit of the human eye is traditionally traced. The human eye is an amazing sensor. It allows to image forms by projecting light emitted or reflected by them using an adjustable lens, the cornea, into a light sensitive concave area, the retina 1. Given such a functioning principle, the limit for seeing small structures is given by the number of sensors pro unit of angle on the retina. In the case of humans, this value is such that two point-like objects can be distinguished from each other if the angle projected on the human retina is bigger than 0.17 $[rad]$ (about a minute of arc). The particular minimal angle of a person is indeed used to diagnose eyes under the name of visual acuity [3].

Put in terms of length a normal human eye can differentiate two dots lying at 0.73 $[mm]$ located one meter in front of it [4]. A more interesting comparison is perhaps this separation at 10 $[cm]$, the common limit for the adaptation of the eye lens. At this distance the eye is capable of distinguishing objects at 25 $[\mu m]$. Everything below that limit melts and becomes invisible.

Microimaging is the science of making invisible things visible. It goes beyond imaging with visible light, but can be applied to almost any signal. In general imaging can be classified according to the signal imaged. This leads to the so called modalities: light imaging, x-ray imaging, γ-imaging, thermal imaging, strain imaging, impedance imaging, flow imaging, etc.

A further classification for imaging in general takes place in space. One dimensional signals do not normally classify under images, so images in spatial sense are divided in two and three dimensional imaging. In between there are several modalities that are indeed not two not three dimensional.

An example is stereoscopic images. The concept behind is that humans can get a three dimensional impression of the world given the fact that they have two eyes. In this binocular view the world is reconstructed from two two dimensional slightly different images into a three dimensional world. The difference in the images arises from the different position and orientation in space of the two eyes or sensors, and thus the different relative position of image components to each other. Systems that try to emulate this are usually not fully three dimensional, since discretize depth when generating the two two dimensional images in case of visualization or when reconstructing the three dimensional information in acquisition [5].

A second example is three dimensional surface imaging. In this case the object imaged has a two dimensional nature (a surface in three dimensions can be completely described by two parameters), but can be acquired in three dimensions if the physical magnitude of interest is acquire on the surface, giving birth to a pseudo three dimensional imaging.

The last classification to be considered in this review is according to the procedure used to achieve the magnification. From 1 it can be seen that in order to magnify distances, a method for increasing angles is needed. This can be achieved by deflecting rays or by miniaturizing the sensor to be used. Since this classification is innate to microimaging and determines directly the resolution of the system, this review will deepen in that classification in the coming sections.

2 Rays and lenses

Probably one of the first devices invented for magnification is the loupe or magnifying glass. Chronicles report its invention in around the thirteenth century [6]. The physics behind the loupe is refraction of waves in boundaries between different media.

According to the wave equation which governs the physics of light, when light reaches a medium change it bends. The angle it is redirected is a function of the incident angle and the quotient of the so called

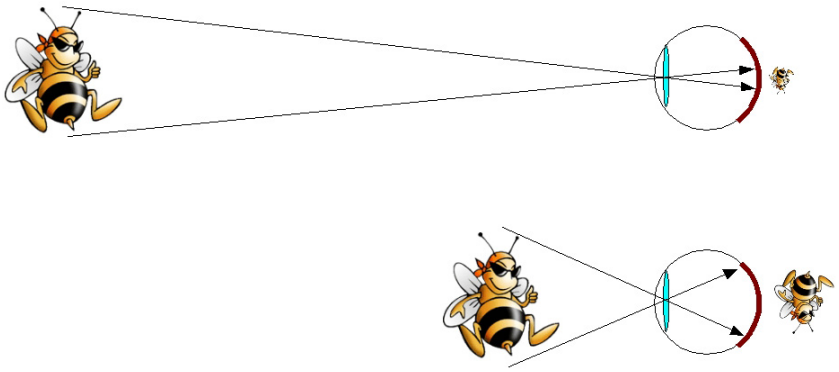

Figure 1: Schematic representation of image acquisition in a human eye. Objects are seen by projecting them on the retina, the sensitive area of the eye. Depending on the distance to the object the image on the retina the object looks bigger or smaller. Bigger objects subtend bigger angles. Closer objects look bigger, because they are in deed bigger!

refraction indexes of the media considered (n_1 and n_2). The refraction index is the quotient of the speed of light in vacuum and the one in the medium of interest. Ibn Sahl derived an empirical law for this process in the late 900, that made famous by Harriot, Snell, Descartes and was latter confirmed using the Maxwell equations [7].

$$\frac{\sin\alpha_1}{\sin\alpha_2} = \frac{n_2}{n_1} \tag{1}$$

In figure 2 the refraction of a light ray in the boundary of two media is shown.

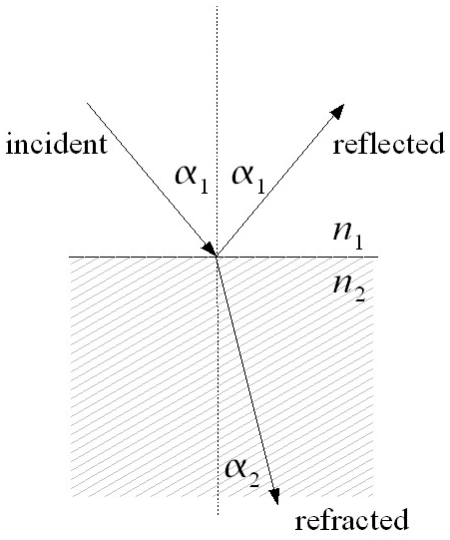

Figure 2: Refraction of light in the interface of two media with different refraction index.

A consecutive construction of different interfaces light rays allow the deflection of rays in almost any given direction and thus enables an increase in angle. Since the Middle Ages, the investigation of different geometries has given birth to lenses, which are not more that transparent bodies for visible light that have a different refraction index as air and thus allow deflecting light. In the case of air and glass convex and concave surfaces give birth to lenses that make light converge or diverge as seen in figure 3.

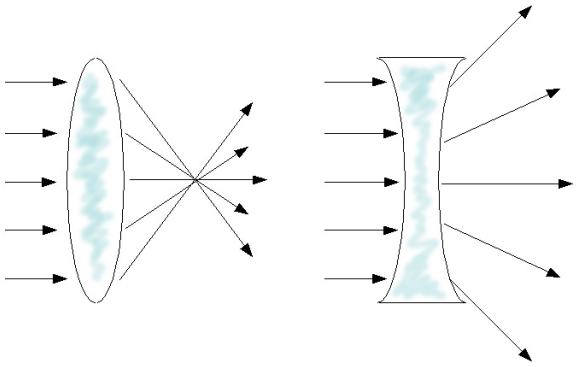

Figure 3: Convex and concave lenses make light converge or diverge given its geometry. In this example the refraction index of the lenses is bigger than the one outside. If this should not be the case the geometries would be switched.

In the case of magnifying lenses figure 4 shows schematically how angles are increased and a virtual magnified image of the object is created on the retina.

Real images can also be generated with simple system for example as shown in figure 5.

In order to improve further more the magnification of loupes and similar systems, Galileo Galilei, Christian Huyghens, Hans Janssen and further scientists developed a two lens configuration in order to amplify an image in two steps in the end of the sixteenth century. The device they created is what we know as microscope. It is not more than two loupes one after the other [8]. Figure 6 presents a simplified version of a bright field microscope or simply microscope.

A modern microscope is not so different in construction from the one presented in figure 6. The most important changes are the inclusion of two further lenses, the condenser lens and the tube lens. The first is used mostly to improve the illumination of the object and the second to enhance the magnification in the eyepiece part [9].

Further types of microscopes are the dark field microscope and the phase contrast one. The first is conceptually just a bright field one with the difference that

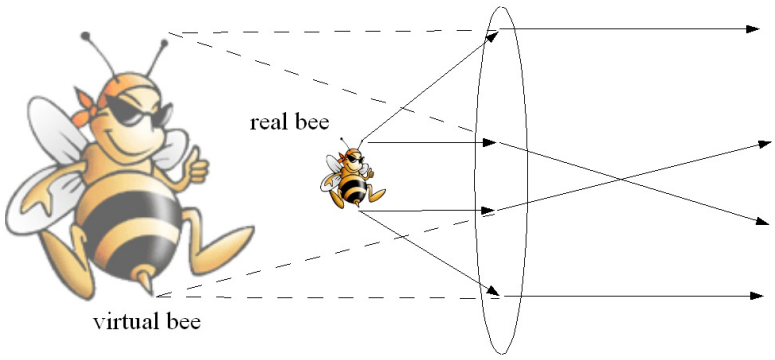

Figure 4: Principle of magnification through lenses in a loupe: The original object is put directly in front of the loupe, which deflects the rays coming from the object. If seen by an eye, these rays seem to come from an object that is not as close as the original, but which is a considerably bigger than the original one. One speaks of a virtual image, because it cannot be imaged with a device like a CCD chip, but is "reconstructed" virtually in the imagination of the viewer based on the input signal (deflected rays).

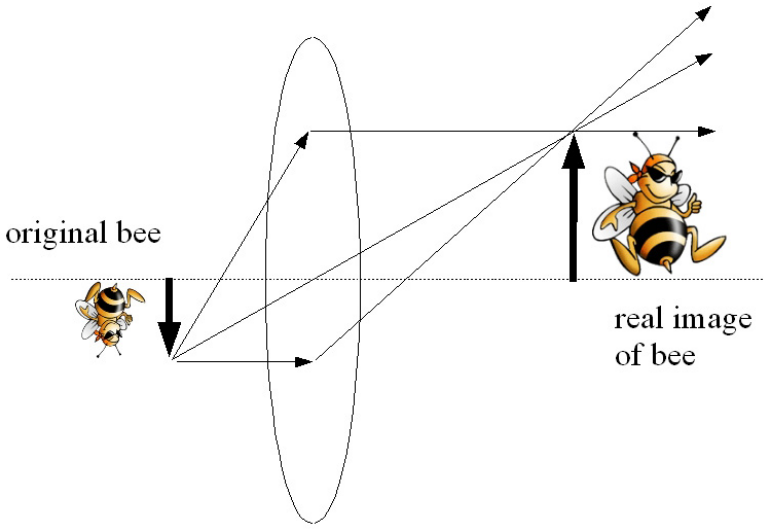

Figure 5: Creation of a magnified real image with a convex lens: In this configuration the rays coming from each point of the bee are deflected and focused back in a plane in from of the lenses. If one would put a photographic film on this plane a magnified image of the bee would appear.

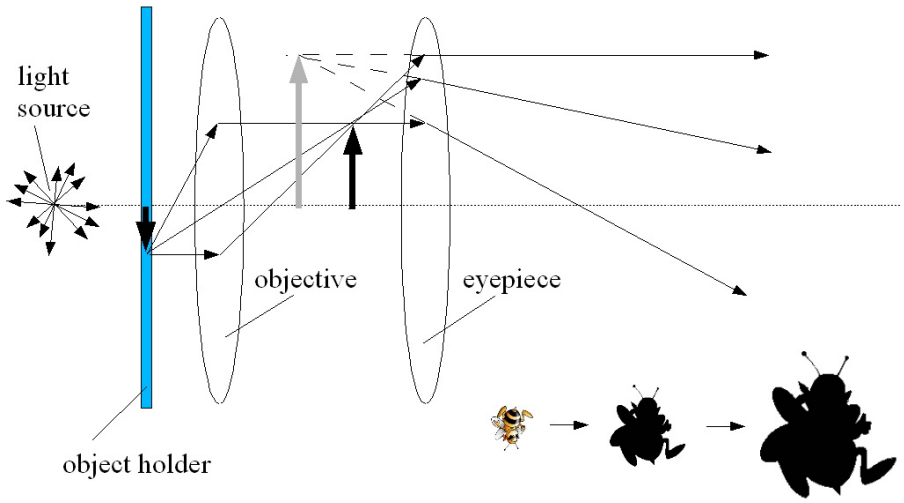

Figure 6: Bright field microscope: The microscope consists in a light source and two lenses, the objective and the eyepiece. The object to be examined (small black arrow) is placed between light source and objective. The objective magnifies the object into a real image (bigger black arrow). The eyepiece plays then the role of a loupe amplifying the bigger image into a virtual image that is even bigger (gray arrow). For a comparison, the bees on the right lower part show the amplification steps from the real object to the virtual one. In general, the magnification of a microscope is the product of the magnification of objective times the one of the eyepiece. Note that, since the object is illuminated from behind, only the contour is seen if it is not semitransparent to light (not the case of bees). The background appears bright (→ bright field microscopy) [9, 10].

it illuminates the object from above creating an image where the object is bright and the background dark (the object does not need to be semitransparent)[1] [10].

The second type, the phase contrast microscope, has a more complex construction and was designed by Frits Zernike in 1934. The idea behind it is to image invisible structures that only make changes in phase on the incident light without coloring them. In order to achieve that, light that was altered by the object (diffracted light) has to be separated from "unaltered" (only phase shifted) light. The "unaltered" light must be then made "visible" and that is all [11]. In figure 7 a schematic model of this microscope is shown and a brief explanation of the functioning is appended. Some preparation is also needed in this case, but at least the coloring and embedding in wax can be skipted.

Further variants of light microscopy are the so called fluorescence microscopy and the luminescence microscopy. The changes for both only are in the light source. In fluorescence microscopy, certain features of the object are marked with fluorescent compounds. When illuminating those compounds with certain monochromatic light they emit in known ranges. The typical example is the use of fluorescent markers that shine in visible light when illuminated with ultraviolet rays. In case of this modality both bright and dark field microscopy is possible using the proper monochromatic light source and eventually a color filter behind the eyepiece to filter out only the fluorescent signal.

Luminescence microscopy is almost restricted to biological gene imaging. Here living organisms are provided with genes that encode proteins like luciferase. This molecule undergoes several chemical paths under which it emits light. A dark field microscope without light source and a proper camera device can thus allow the implementation of such a system.

Lens based systems were till the end of the 19th century the only magnification devices used. In fact, the concept was only used for light for a long time. Nonetheless, it can be generalized to any kind of ray. The natural extension of it would be other electromagnetic waves and acoustic waves, but also particle rays. These ideas have been introduced in the last years mostly to overcome the limitation of optical magnification.

Before going into details in those systems, one needs to consider the restriction of lens systems. Given the brief introduction in the last paragraphs, there seems to be no limit to magnification, but the construction of adequate lenses. Restrictions given to construction issues are thinkable[2], but a fundamental limitation only appears when considering diffraction.

Although for macroscopic and even "big" microscopic levels light may be considered as a ray, if one pushes up the magnification its wave character begins to show. In deed there is a limit above which the magnification of the object studied does not add further details to the image. One speaks of "empty" magni-

[1] In bright field microscopy the object needs normally to be prepared in order to see more than the contour. Typical preparation includes the embedding in wax or the freezing (in order to harden the object), the cutting in thin layers (in μm range) and the coloring of the structures of interest. This is a major limitation for studies of fragile tissues, living tissues or chemical unstable structures. Dark field microscopy does not require this steps, but requires considerably more illumination.

[2] Some of the most important optical restrictions are spherical aberration, chromatic aberration, astigmatismus, coma, etc. [12]

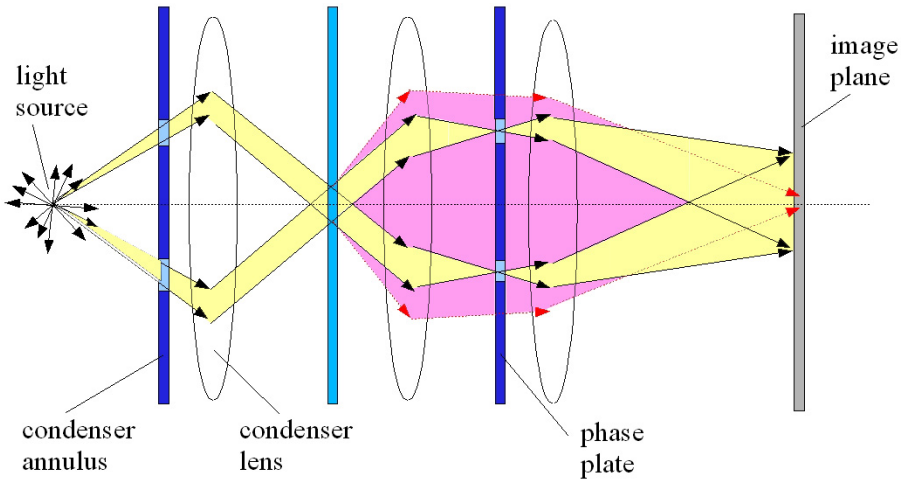

Figure 7: In the phase contrast microscope the object is illuminated from behind, through a condenser annulus, which is an absorbing structure with a ring aperture, and a condenser lens. The illumination thus takes place only from a certain angle from the center line of the system. As light interacts with the object a diffuse light cone is generated (shown by the red arrows and the space in between (rosa or yellow if coincident with only phase shifted light). Light that passes through transparent objects and is only shifted in phase continues with the same trajectory (black arrows and the yellow area in between). The light, both diffuse and only phase shifted is bent by the objective and generates an image of the object directly over the phase plate. The phase plate is a matching structure to the condenser annulus and modifies the only phase shifted by retarding it to achieve a phase difference of half a wavelength. This phase difference causes then the only phase shifted light to interfere with the diffuse light producing shadows on the image exactly where the transparent structures are located (real image of the transparent structures) [11].

fication. This phenomenon happens when the effects of diffraction begin to show. Diffraction is a medium dependent deflection of light in all directions. In practice a magnification system where diffraction is visible shows real points as a group of concentric circles centered at the position the ideal image of the point would be (Airy patterns). Figure 8 shows two points of an image after a magnification system. Airy patterns can be clearly seen.

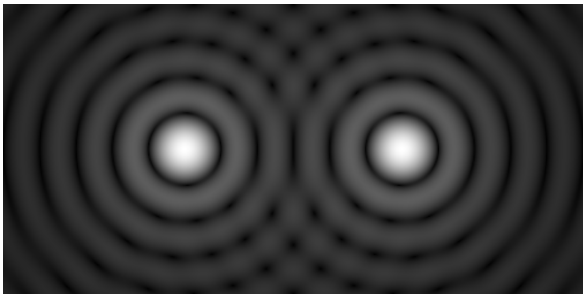

Figure 8: Two points far away from each other imaged by a real magnification system.

The problem is that this fact limits resolution. Resolution was mentioned above and is the capability of distinguishing as different entities two points. It is usually given by the minimum distance (d_{min}) between the points in order to consider them separate entities. In case of a magnification system like the one used for figure 8, a critical case would be figure 9. Here the diffraction effects cause both points to melt and so make the space between invisible.

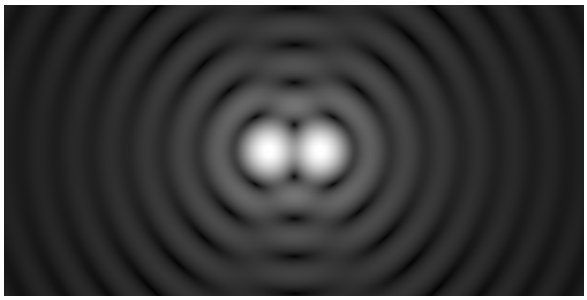

Figure 9: Two points close to each other imaged by a real magnification system.

A good approximation for the resolution is given by the following equation (taken from [13, 14]):

$$d_{min} = \frac{0,61\lambda}{N.A.} = \frac{0,61\lambda}{n \sin \alpha} \tag{2}$$

where λ is the wavelength of the light used and $N.A.$ is the numerical aperture of the system, which is function of the refraction index of the medium between object and objective n and the angle of the light cone between object and objective lens α.

This formula puts into the spot light the fact that for small structures differences in color may case distortion on the image. This problem is one variant of the so called chromatic aberrations and can be dealt with special correction or by using monochromatic light [12].

In case of optimized light microscopes this restriction puts a stop near 200 $[nm]$ under optimized conditions [13, 14]).

Microscopy nonetheless has gone further. The trick is simple: abandoning light for smaller wavelength rays, for example ultraviolet light, x-rays and γ-rays. A second option is to abandon the electromagnetic waves and try other kinds of rays. Only two options come in question: sound and particles. Sound is also a wave. The problem is nonetheless that given its low velocity the wavelengths are many orders of magnitude above light making it not so interesting.

Particle rays are on the other hand of great interest. Based on the derivation of De Broglie that connects matter and waves into matter waves, one can calculate a wavelength for particles. The formula is:

$$\lambda = \frac{h}{mv} \tag{3}$$

where h is Planck's constant, m the mass of the particle and v the speed of the particle ray.

Without getting into details, this gives wavelengths that are far below electromagnetic ones even for slow motion rays and thus promises an increased resolution. The problem is, however, how to make those rays visible and even worse, how to design lenses for them.

Staying still in the world of electromagnetic waves, UV lenses and x-ray lenses have been developed and are still in development nowadays. For UV quarz lens is the common solution. It is nevertheless expensive. For x-rays lenses are also not conceptually different than the optical ones, but require a lot of engineering for useful implementation (see [15, 16, 17] for examples), so costs and still construction issues limit their application.

Particle lenses, if one can speak about such a thing, exist for charged particles. In deed they are in almost any house hold of Europe. One only needs to take a look at a television. An example is shown in figure 10. Of course, imaging lenses have to to fulfill strict requirements and television devices not necessarily.

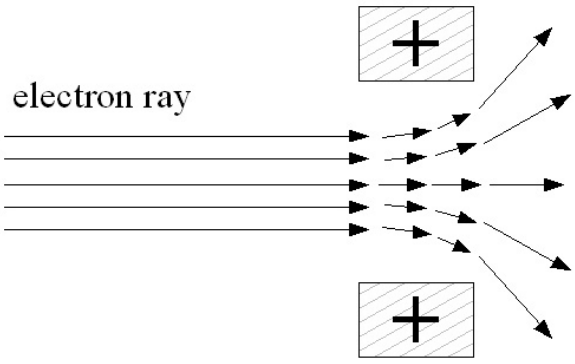

Figure 10: Example of an electrostatic lens for an electron ray.

In general charged particle rays can be deflected by electric or magnetic fields (being the last the most commonly used) [18]. Both modalities have been used for the construction of complex particle ray based microscopes, being perhaps the most famous example the transmission electron microscope by Ernst Ruska in 1933 [19]. A transmission electron microscope consists of a layout and a functioning similar to the one seen in figure 11. A preparation step of the object is also needed here[3]. Moreover, electrons are invisible to the human eye. The use of luminescent screens, faceplates and similar devices capable of emitting visible light on the contact with the electron beam is a common solution. The resolution for current standard electron microscopes achieve lies below 2 $[nm]$ and even less for the newest high resolution modalities (down to 78 $[pm]$ (atoms!) [18]).

3 Miniaturization and scanners

In the last section a brief introduction to magnification based on lens systems was presented. Lens based magnification is nonetheless not the only option. An interesting idea is the miniaturization of the sensor. The concept is more or less like this: if the limit of resolution for humans is 25 $[\mu m]$ what if an imaging system is designed, such that the distance between sensors is smaller than 25 $[\mu m]$. In case of a CCD or CMOS light imaging system, this would allow an acquisition of a resolution higher than the one reachable by the human eye and the magnification could be achieved simply by displaying the collected image on a screen bigger than the image itself by a factor of at least 25 $[nm]$ / (sensor-sensor distance).

Now the question is if such small sensors can be implemented. The answer is yes and no. For CCD sensors for instance the sensor length of standard camera sensors is about $2.5-3.5$ $[\mu m]$ and for CMOS $7.5-11$ $[\mu m]$ [20]. Nevertheless, the magnification thus achieved is too low.

An interesting idea arises from the fact that in many applications the signal can be considered constant in time for small time intervals. If that is the case the separation between sensors can be virtually decreased almost to zero. This is achieved by using unidimensional small sensors and scan the area to be imaged. Figure 12 shows schematically how a two dimensional image is obtained from a given variable in space. This idea is nonetheless also applicable for three dimensional imaging.

Requirements for a correct implementation of this idea are small sensors and a synchronized recording of position in x and y for two dimensional imaging and also z for three dimensions, as well as fine and precise positioning system of the sensor. Both depend on the type signal to be acquired. A great advantage of this modality is the possibility of recording almost any signal. Furthermore objects do not need to be semitransparent nor treated specially as a general rule. Disadvantages arise from the fact that the object needs to be scanned and for many signals, this is only possible by direct contact.

In order to enlighten the principle of scanning systems the example of the scanning electron microscope

[3]The preparation of objects for transmission electron microscopes is conceptually similar to the one for light microscopy, but more complex. This limits the applicability of such devices.

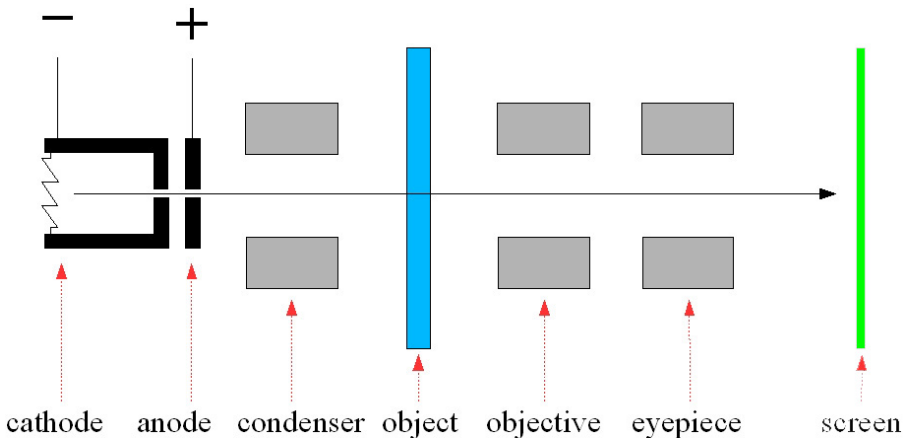

Figure 11: In a transmission electron microscope, an electron beam is generated by accelerating electrons in a high voltage electric field ($5 - 50$ $[kV]$ normally). The cathode is heated in order to free the electrons which fly guided by a Wehnelt cylinder to the anode. The anode has a small orifice in the center which allows electrons in flying close to the symmetry axis to continue the flight. The condenser lens is then responsible to adequate the electron ray and project it on the object which has been previously prepared. The electrons that pass the object (in order to be able to image more than the contours of the object it must be "semitransparent" to electrons) are then deflected to amplify the image in the objective and eyepiece lenses. The ray carrying the information is converted in the screen (faceplate or luminescent screen) into visible light [18]. The whole system works under strict vacuum conditions.

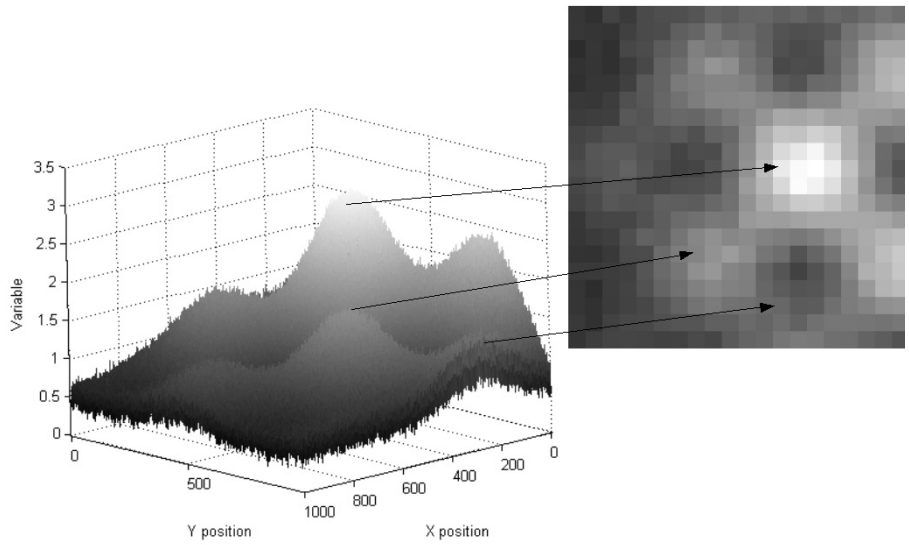

Figure 12: Idea of two dimensional scanning for imaging. The variable to be imaged is shown as surface being the z coordinate the value of the variable. By locating a sensor at known a given known position given by x and y, the variable can be recorder and the value can be represented at the acquired position on the image plane. Here three points are shown both on the surface and the image.

is shown in figure 13.

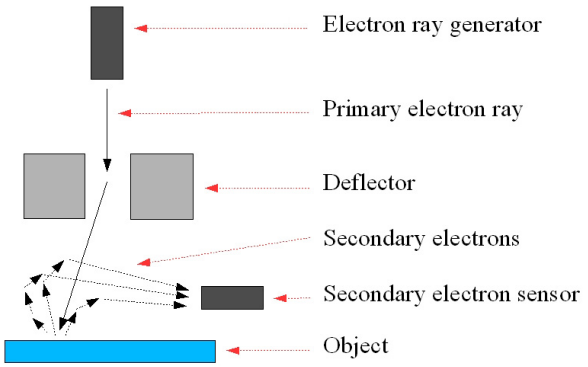

Figure 13: Scanning electron microscope: In this type of microscope a thin ray of monochromatic electrons (primary electrons) is focused on a single point of the object by means of a deflecting field in both x and y direction. The ray interacts with matter producing among others, low energetic secondary electrons that contain mostly information on the surface of the object at the focused points. Those electrons are collected by a sensor, that is usually capable to analyzing them according to their energies. Once the whole object has been scanned the image is shown on screen while process continues refreshing constantly the old measurements.

Scanning microscopy is present in almost all new procedures. An interesting case is the so called tunnel effect microscope introduced in 1981 by Gerd Binnig and Heinrich Rohrer. In this modality the surface of an object is imaged up to huge precision by scanning it with a very sharp tip. A voltage is applied between object and the scanning tip. This voltage raises the energy levels of electrons in the object up to a point where they can tunnel to the tip and thus generate a signal. The probability of tunneling decays exponentially with distance, making it possible to estimate the distance between tip and object and thus the topology of the surface. The resolution of such a system lies close to 200 $[pm]$ (atoms!) [21]. Figure 14 shows the principle of functioning.

Further modalities of interest are EDX (energy dispersive x-ray spectroscopy), FIB (focused ion beam), AFM (atomic force microscope), etc.

4 Mixtures, the present and limits

In the preceding sections the principles of lens and scanning microscopy were introduced. In this last part of the review we include interesting examples where both modalities are combined.

The first example is confocal laser scanning microscopy. In this modality the object is scanned in three dimensions by a laser beam with proper geometric form such that it focuses in the current point being scanned. The reflected light is them sensed after filtering out the light out of focus. The point of focus is simultaneously recorded and a three dimensional image is achieved [22]. Figure 15 shows the schematic construction and the functioning principle.

A second example is scanning transmission electron microscopy here transmission electron microscopy is applied in several points of a raster allowing the use of thinner beams. The transmitted electrons are collected behind the object as done in the non scanning modality and the position is stored.

Variations and combinations of the procedures presented are common nowadays. The future will belong to them. The question is however if limits will be pushed further down or if the cost will tie the pretensions of scientists still for some years. Beyond the limit was already fixed by Heisenberg more than eighty years ago. It lies were uncertainty and invisibility become synonyms.

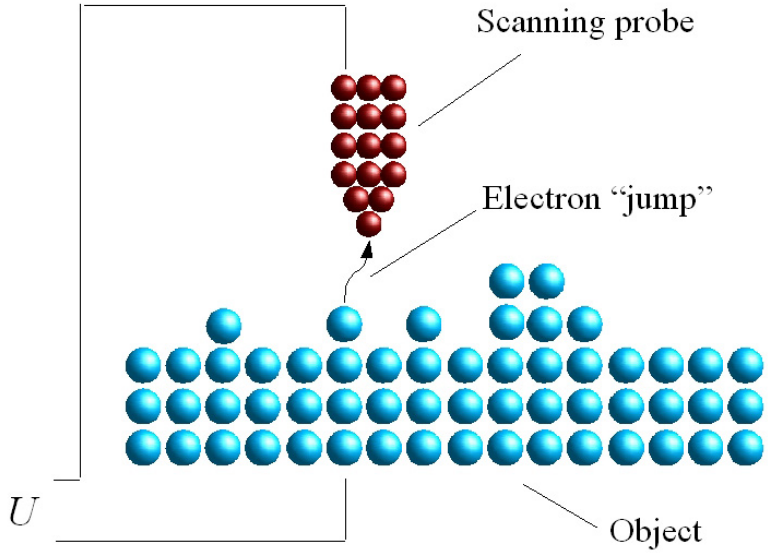

Figure 14: Scanning tunneling microscope: A very thin (only a couple of atoms) scanning probe is used to scan the surface of an object. A voltage is put between probe and object. Electrons on the surface of the object can tunnel the energy barrier and jump to the probe. Those events are counted. The probability of such and event is related by an exponential law to the distance between probe and object. Thus by measuring the current the distance can be estimated. By repeating the procedure over the whole surface an image of it at atomic level is produced [21].

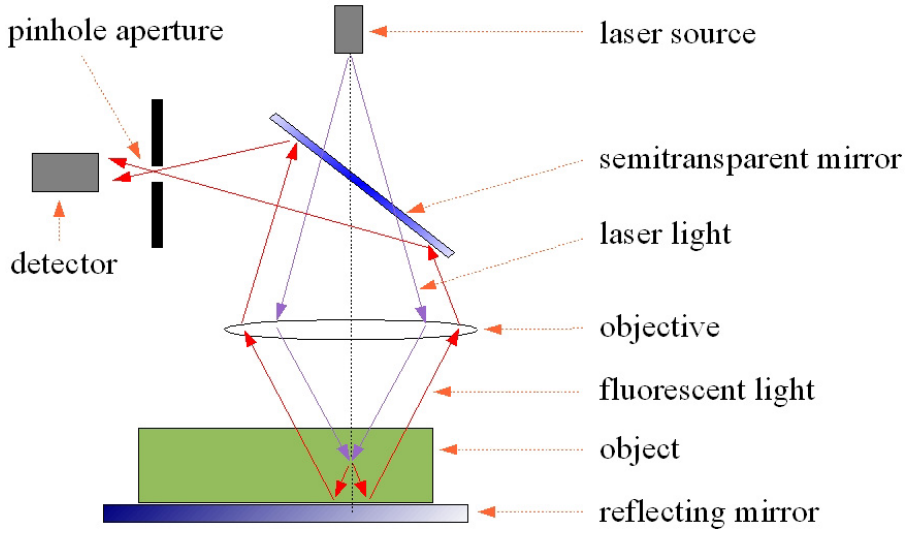

Figure 15: Confocal laser scanning microscopy: A monochromatic laser beam is focalized at a certain point of the object to be studied. The laser light excites the previously fluorescent marked structures to be analysed and makes them send fluorescent light. Both the laser light and the fluorescent light are reflected on a reflecting surface above which the object lies being sent back to the light source. The fluorescent light is then reflected again in direction of the sensor by a semitransparent mirror that allows laser light to pass but reflects fluorescent light. The reflected fluorescent light faces then a pinhole aperture which is located in a way such that only light coming from the focused point passes. A detector, commonly a photomultiplier, obtains then the fluorescent information of uniquely the point focused. The relative positions of the parts of the system can then by modified to scan another point of the object in three dimensions [22].

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