

## OPTICAL AND ELECTRON MICROSCOPY

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### Contents

1. Introduction
  2. Optical microscopy
    - 2.1. Historical Development
    - 2.2 Principles of Optical Microscopy
      - 2.2.1 The Standard Compound Microscope Configuration
      - 2.2.2 Diffraction, the Abbe Theory of Imaging, Resolution Limits and Aberrations
      - 2.2.3 Sample Preparation
      - 2.2.4 Image Contrast and Interpretation
  3. Electron microscopy
    - 3.1 Historical Development
    - 3.2 Transmission Electron Microscopy
      - 3.2.1 Why use Electrons for Microscopy?
      - 3.2.2 The Instrument
      - 3.2.3 Principles of Imaging and Diffraction in the TEM
      - 3.2.4 Sample Preparation
      - 3.2.5 Principal Applications of TEM
    - 3.3 Scanning Electron Microscopy
      - 3.3.1 The Instrument
      - 3.3.2 Contrast in SEM Images
      - 3.3.3 Sample Preparation
      - 3.3.4 Principal Applications
    - 3.4 Analytical EM
  4. Hybrid and derived instruments
    - 4.1 Confocal Microscopy
    - 4.2 Scanning Transmission Electron Microscopy (STEM)
  5. Current trends and perspectives
  6. Conclusions
- Glossary  
Bibliography  
Biographical Sketch

### Summary

In this chapter, the history, principles and applications of optical and electron microscopy are reviewed.

The standard optical microscope, which is still the most widely used type of microscope, has remained essentially unchanged for more than a century, since the theoretical resolution limit of about 200nm (set by the wavelength of visible light) was attained in the 1880s. The instrument consists essentially of a combination of two lenses, an objective and an eyepiece, the product of whose magnifications gives the magnification of the final image. It is an irreplaceable tool in the biology or biomedical laboratory for the study of cell and tissue structure and for general biopsy work. It is also very widely used in the materials sector. Modern developments do exist, including confocal microscopy, yielding high quality 3-D information.

The transmission electron microscope (TEM) provides the highest resolution images available, with atomic resolution being routinely achieved for a wide variety of materials. The technique is analogous to its optical counterpart in the transmission mode (light or electrons being transmitted through the sample before being used to create the image. The very high quality performance of the instrument comes at the price of rather extreme specimen preparation requirements, particularly in the life sciences, and very complicated contrast mechanisms, making image interpretation difficult, particularly in the physical sciences. The imaging capabilities of the instrument are complemented with easily obtainable diffraction patterns for structure studies and spectroscopic techniques for chemical analysis of the specimen.

The scanning electron microscope (SEM) combines, at least partially, the ease of use of the optical microscope with the analysis capacities of the TEM. The imaging principle of the instrument is very different from those of the other microscopes described here. The objective lens does not form an image of the sample, but rather a fine probe that is scanned across the sample surface. The image consists of the variation, as a function of the probe position, of some signal generated by the probe's presence. This signal is most often the secondary electrons emitted by the sample under the probe. The SEM can observe bulk samples several centimeters in size and is consequently more flexible in its applications than the TEM. The highest resolution attainable is however somewhat more modest — of the order of a few nanometers. Applications are extremely varied across the life and physical sciences.

## 1. Introduction

From the simple magnifying glass to the most advanced electron microscopes, there exists today an ever-expanding range of instruments whose principal function is to produce a magnified image of a small object. In fact, the sheer number and diversity of the techniques available renders a complete description of all of them beyond the scope of this chapter. Here we will limit ourselves to a presentation of two of the three main families of instruments: optical, and electron microscopes. The highly surface-sensitive "near field" or "scanning probe" microscopes are covered in *Optical Sources and Detectors*. Important sub categories and/or techniques will be included where possible.

Optical microscopy may be defined as the formation of a magnified image of a small object, using visible light as the illumination. It is still the most widely used of these three families, mainly because of its cheapness and simplicity. However, it also possesses advantages and unique features which are not available from non-optical instruments. In addition, there have been recent developments such as confocal

scanning optical microscopy, which have increased its capabilities in some areas. But the essential design of the standard instrument has not changed for over a century; an objective lens forms a magnified image of the object, which is then further magnified by the "eyepiece" lens. The product of the magnifications of objective and eyepiece gives the final magnification. Optical microscopes are used widely for the observation of biological sections, for metallographic observations and for quality control in a wide variety of areas, notably in the electronics industry. Although training is necessary for a microscopist to exploit all the possibilities of the instrument, basic operation for routine visual checking is very simple and can be performed quickly. The principal disadvantage of the optical microscope is the natural resolution limit, which restricts the minimum size of detail that can be seen to about 0.2 microns. This limit is imposed by the wavelength of visible light (which is of the same order — around 0.5 microns) and thus cannot be overcome by improving the optical components of the instrument. One way of overcoming this limit is to use shorter wavelength light, i.e. ultraviolet light or even X-rays. X-ray microscopes constitute an active area of research and can yield images of high resolution, but the focusing of X-rays is problematic and the instruments currently still have severe limitations. We will not further discuss their use here.

Another solution is to use a different type of radiation altogether to "illuminate" the object. Quantum physics describes how sub-atomic particles, such as electrons, protons and neutrons, can be treated as waveforms (just as visible light may also be considered to be particulate in nature). This so-called wave-particle duality means that a microscope can, in principle, be constructed using object "illumination" by any type of particle, and its resolution will theoretically be limited by the wavelength of the particle's associated waveform. In practice, the best particles to use for this purpose are electrons, which can be readily produced in a collimated beam and also can be accelerated and focused by electric and magnetic fields. An electron accelerated by an electrical potential of several hundred kilovolts (kV) has a wavelength of a few picometers ( $10^{-12}$  m). A microscope capable of resolving detail on this scale would reveal considerable structure in the interior of individual atoms. In practice, the best high-resolution electron microscopes (HREMs) currently resolve details of around 0.1 nm in size. The theoretical limit is far from being reached because of lens aberrations and instrumental instabilities. The performance available nevertheless allows one to achieve atomic resolution — atomic positions can be seen, even if the internal structure of the atom is not visible.

There exist two main families of electron microscope. The transmission electron microscope (TEM) is an instrument that achieves the highest resolution. (The terms TEM and HREM are to some extent interchangeable, although the TEM is not used exclusively for high-resolution observations). The TEM, as its name implies, uses electrons that have been transmitted through the object to form the image. Its principal drawback is the requirement to use specimens in the form of very thin (10 — 100 nm) sections, which can be very difficult to produce, and which may not be representative of the larger piece of material from which they are taken. The scanning electron microscope (SEM) is a more flexible and versatile instrument than the TEM and can be used to image comparatively bulky objects, relying as it does on electrons emitted from the sample surface to form the image. Specimen preparation is thus generally more straightforward than for TEM, as is operation of the instrument and interpretation of the

images. The resolution achievable is however limited to a few nanometers, so that detail on the atomic scale cannot be observed.

We will now describe each of these groups of microscopes in more detail. The fundamental principles and the major applications of each instrument will be presented in a concise way. Mathematical treatments will be kept to a minimum. Full, rigorous treatments of the various imaging-forming mechanisms can be found in the textbooks and monographs listed in the bibliography.

## **2. Optical Microscopy**

### **2.1. Historical Development**

There appears to be no sure evidence for the use of glass lenses to magnify objects before about the end of the 13<sup>th</sup> century AD. At this time the use of spectacles became widespread in Italy. The first compound microscopes appeared in Holland around the end of the sixteenth century and their invention is usually attributed to the spectacle maker, Hans Jansen. However, Dutch microscopist Antoni van Leeuwenhoek is also often cited as the father of microscopy, since the single-lens instruments he pioneered from about 1670 (essentially high-powered magnifying glasses) were superior in performance to the compound instruments available until about or even after 1800. In the first few decades of the 19<sup>th</sup> century, lenses corrected for chromatic and spherical aberrations were being developed, notably by the British amateur scientist J.J. Lister. This work culminated in the designs of German physicist Ernst Abbe, who also formalized the theory of image formation in 1877. By 1880, instruments were in use that had attained the theoretical limit of resolution for light microscopy. The most significant developments in the 20<sup>th</sup> century were probably the development by Zernike in the 1930s of "phase contrast" microscopy, allowing details to be seen in thin transparent sections which are almost invisible using standard microscopy, and confocal microscopy, yielding 3-dimensional images.

### **2.2 Principles of Optical Microscopy**

#### **2.2.1 The Standard Compound Microscope Configuration**

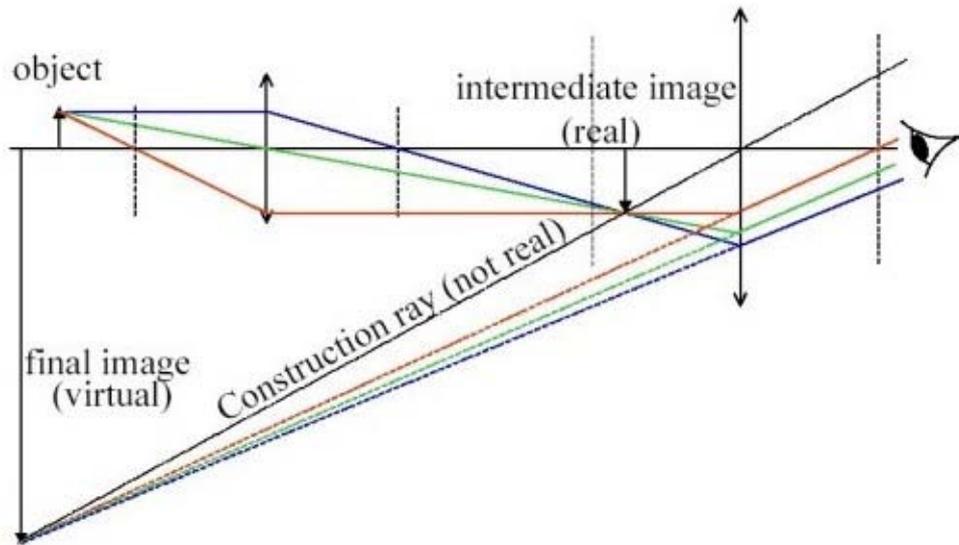


Figure 1: The compound microscope

The description which follows assumes some basic knowledge about lenses and their image forming properties, which can be found in *Imaging and Characterising- Trace Element Analysis*. A typical bench-top optical microscope consists essentially of a combination of two lenses (Figure 1). The objective lens forms a real, magnified image of the object. This real image serves as an object for the second lens, the eyepiece, which forms a virtual magnified image of it. The observer looking through the eyepiece thus sees this inverted virtual image whose magnification compared to the original object is given by the product of the magnifications of the two lenses.

In reality, in a modern instrument, the eyepiece and the objective each consist of a group of several lenses combined in such a way as to minimize aberrations and other distortions. But these groupings are usually considered (and sold) as single lenses with a given focal length and numerical aperture (see next section).

In addition, the eyepiece may be replaced with a camera consisting of a lens positioned so as to form a real final image in a plane containing a viewing screen, a photographic film or a digital image-recording device. The magnification in this final image plane is still given by the product of the magnifications of the two lenses.

The dispersion of the light rays caused by the magnification process means that the object must be brightly illuminated if the final image is to be visible. Most microscopes are fitted with condensing illumination systems, which can focus light into a small spot on the sample surface at the point to be viewed. This light may impinge from the opposite side to the objective, in which case transmitted light is used to form the image (this is often the case for biological thin section samples). If it impinges from the same side as the objective, then the reflected light is used (this is the standard configuration for metallographical observations).

### 2.2.2 Diffraction, the Abbe Theory of Imaging, Resolution Limits and Aberrations

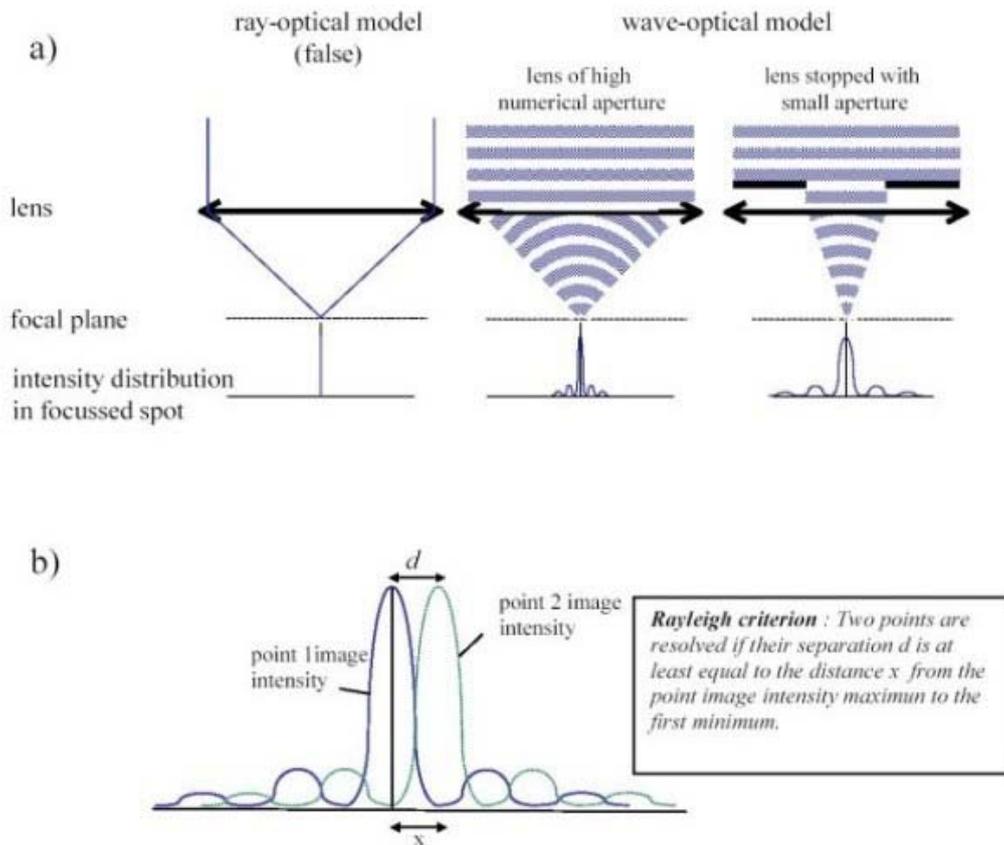


Figure 2: a) Focusing of parallel light to a point b) Rayleigh criterion for resolution of two points

The ray optics model presented above ignores the wave-nature of light. In fact, parallel light rays arriving at the lens will not be focused to an infinitely small point. Light waves consist of oscillating electric and magnetic fields, whose strength or "amplitude" varies as a function of position and time. At a given moment in time, the electric field of a parallel beam of light may be thought of as a "plane wave" — a series of peaks and troughs in amplitude running parallel to the direction of propagation. (The amplitude is thus constant in directions perpendicular to the propagation direction). The distance between two peaks is the wavelength  $\lambda$  and for visible light it is around 500nm. A property of waves is that they interfere with each other; the crest or peak of one wave coinciding with the trough of another of equal amplitude will result in a canceling out of the amplitudes of both waves, whereas two peaks coinciding doubles the amplitude. This means that many plane waves arriving towards a point from different angles combine to yield complicated interference patterns, with localized intensity maxima and minima. However, Abbe showed that no combination of waves of a given wavelength can be found which concentrate all the intensity into a zone whose size is less than about  $\lambda/2$ . This size is achieved if plane waves of equal amplitude arrive towards a point from all directions.

The action of the lens can be seen as breaking up the incident plane wave into a range of plane waves all arriving at the focal point from different angles. The greater the angle subtended by the lens at its focal plane, the greater is the range of angles contributing

(Figure 2). A plane wave arriving at a lens, will thus be focused into a disc (known as the Airy disc) at least  $\lambda/2$  in diameter, no matter how good the lens is. But this size will increase if the lens diameter is small compared to its focal length, or if a small aperture is centered on the principal axis close to the lens. In addition, there will subsidiary maxima in the form of rings around the central disc caused by the interference of waves scattering or "diffracting" from the edge of the lens, or aperture if there is one. (The terms "scattering" and "diffraction" are sometimes used almost interchangeably, but most authors reserve the use of "diffraction" for cases in which the scattered waves interfere with each other to cause intensity peaks and troughs).

Similarly, light emitted or reflected from a particular point on the object will be focused not to a perfect point but into a blurred disc at least  $M\lambda/2$  in diameter, where  $M$  is the magnification (image size /object size). By a convention known as the Rayleigh criterion, it is said that two points are separately resolved, if the distance between the intensity maxima in their images is at least equal to the distance from the maximum to the first minimum in one of the images (see Figure 2b). This distance, which is also at best approximately  $M\lambda/2$  defines the resolution limit of the microscope. (although it can be surpassed in some cases using modern computational techniques of image treatment). The resolving power of the lens approaches this theoretical optimum as the ratio of its diameter to its focal length increases. Conventionally, objective lenses are classified in terms of their numerical aperture (NA) which is defined by the relation

$$NA = \frac{\sin \alpha}{n}$$

where  $\alpha$  is the half angle subtended by the lens at its focal point and  $n$  is the refractive index of the medium between the lens and its back focal plane — see Figure 3.

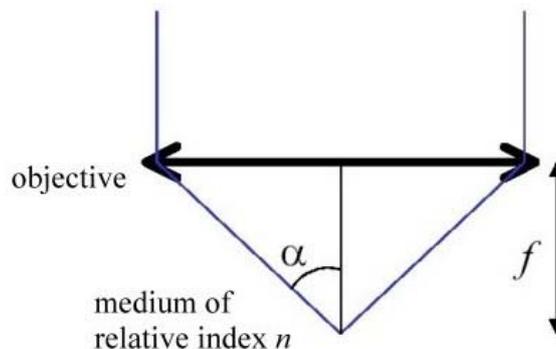


Figure 3: Definition of the numerical aperture, NA

Modern objectives combine very short focal lengths with wide acceptance angles, leading to values of  $\sin \alpha$  approaching 0.9. This is in practice the maximum value that can be achieved in air ( $n = 1$ ) but "immersion lenses", designed to function with a film of high refractive index ( $n = 1.5$ ) oil occupying the space between lens and sample (or cover slip) can increase NA to about 1.4. Such a lens will reproduce detail down to the scale of about  $0.2 \mu\text{m}$ .

In addition to the limits imposed by the nature of light, the lenses may also be imperfect in their operation. Important defects include: spherical aberration, which means that

rays impinging on the lens at large angles to the principal axis are focused in a different plane from those arriving parallel to the axis, chromatic aberration, which means that light of different colors is focused in different planes, field curvature which means different parts of the object are focused in different image planes and image distortion, which means that straight lines in the object appear skewed in the image. Most of these defects can be almost completely eliminated by careful lens design, but an objective lens in which they are all minimized will be very expensive. Users may prefer to consider the imaging defects that are important for their applications and invest in lenses which are well corrected only for these.

### **2.2.3 Sample Preparation**

The degree to which specimen preparation is necessary depends to a great extent on what type of sample is under observation. We will just cite a few examples from the rather vast range of methods used. For metallographical studies, either high quality polishing or a chemical etch may be necessary. Chemical etching can show up features such as grain boundaries and dislocation sites. However, the sample must often be observed as received if the property of interest is not to be altered (the surface condition after an oxidation process, for example). Biological tissue must usually be cut into slices, a few microns thick by means of a microtome, and either stained or marked with fluorescent compounds to yield higher contrast. Individual objects such as cells can be deposited on glass slides by various means and are also often stained. Much of the art of the optical microscopist is in the selection of staining/marketing techniques that will show up the type of matter of interest in a particular study. In general, care taken and time spent carefully preparing the specimen is amply rewarded in terms of the quality and interpretability of the observations.

### **2.2.4 Image Contrast and Interpretation**

The final image consists of variations in the local intensity of light in the image plane. These variations are known as contrast. One of the most important skills of the microscopist is to be able to interpret the contrast correctly. Only in this way can sensible deductions be made about the nature of the sample. In standard optical microscopy, the contrast is due mostly to absorption and scattering of the light by the sample. Light which is absorbed by the sample, or which is scattered (or reflected) outside the acceptance angle of the objective lens or its aperture will not be available to form the image. Consequently, parts of the object that absorb strongly will appear dark in the image. Parts of a sample that have been marked with a molecule that fluoresces under the illumination will always appear bright (since some of the emitted light will be axial). In the transmission geometry, parts of the sample which scatter strongly to high angles will also appear dark if the incident illumination is axial (along the principal axis of the instrument — Figure 4a). Such an image is called a "bright-field" image, since in the absence of a sample the field of view appears uniformly bright. A "dark-field" image is obtained when the incident illumination is off-axis (Figure 4b). In this case only those parts of the sample scattering light back into the axial direction appear bright. Whether an object appears bright or dark is thus not an inherent property of the object itself, but a consequence of how it was prepared and how we are observing it.

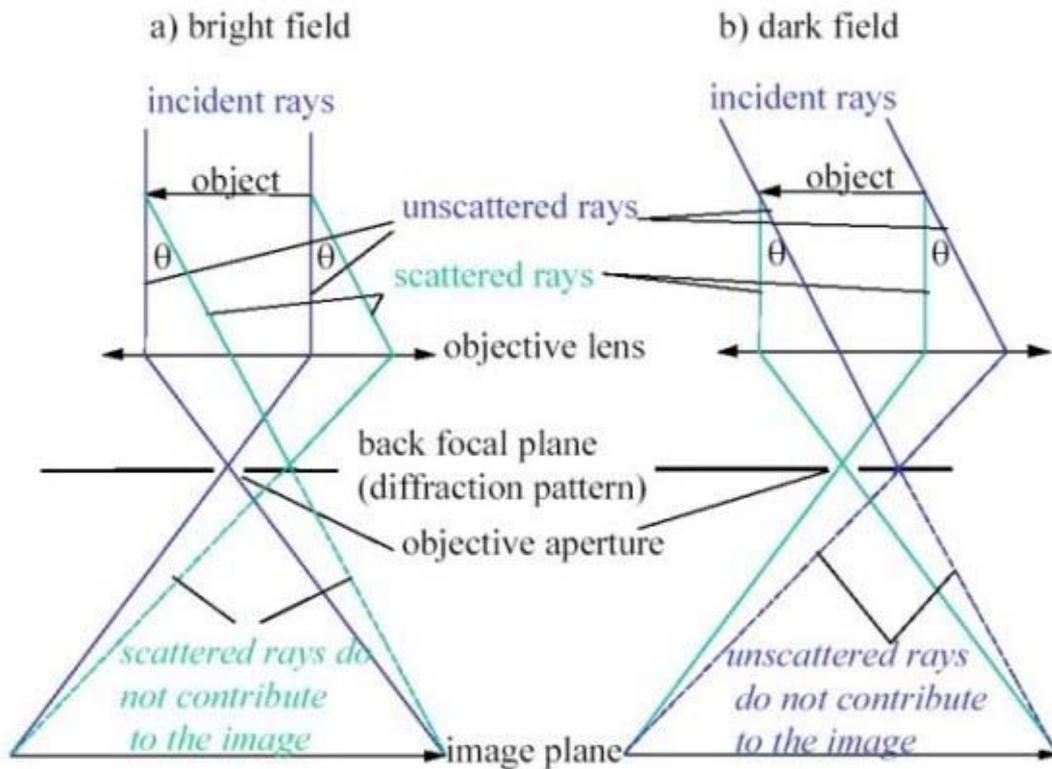


Figure 4: Ray diagrams for a) bright-field and b) dark-field image formation.

Contrast can be increased in a bright field image by placing an aperture in the objective back focal plane, limiting the scattering angle of the light passing through to the image plane. In this way, objects that scatter even to small angles will appear dark in bright field. There is however a price to pay in terms of resolution, because of the consequent reduction in effective numerical aperture. *Phase contrast microscopy* is a technique for increasing the contrast from unstained thin biological specimens (which absorb and scatter relatively little) without sacrificing resolution. Light passing through a thin section of transparent material is not absorbed, but is retarded compared to light passing through the air, usually by one quarter wavelength, or  $\lambda/4$ . The idea of phase contrast is to increase this retardation or *phase difference* to  $\lambda/2$  (see Figure 5). In that way, light having passed through the sample will interfere destructively with a reference beam of unretarded light, and the corresponding part of the sample will appear dark compared to regions in the field of view where there is no matter present (e.g. holes).

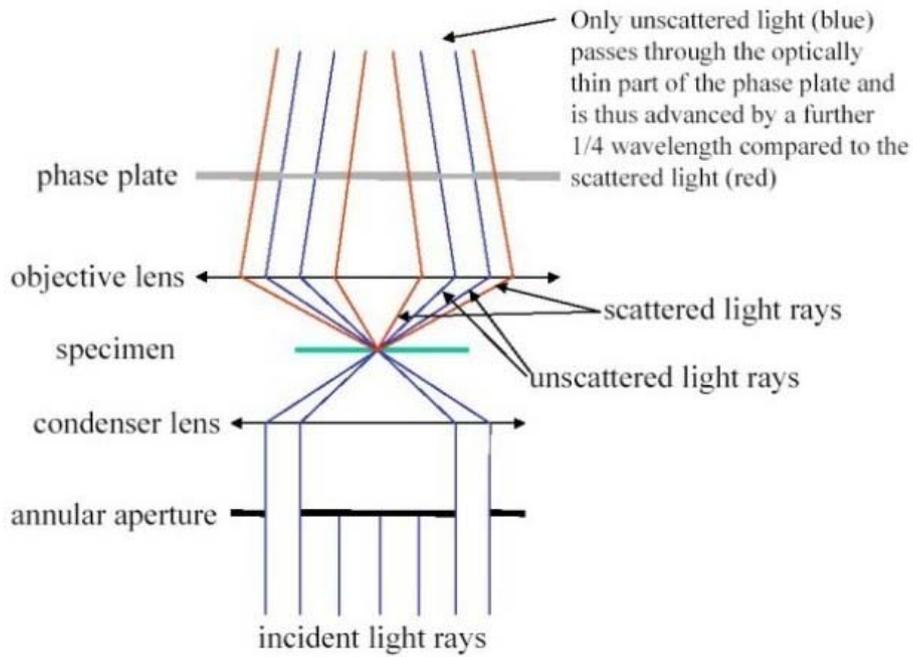


Figure 5: Principle of phase-contrast imaging.

An example of a phase contrast image of an unstained object is shown in Figure 6. This is just one of a number of contrast-enhancing techniques available to microscopists. Users must decide which method is best suited to their requirements.

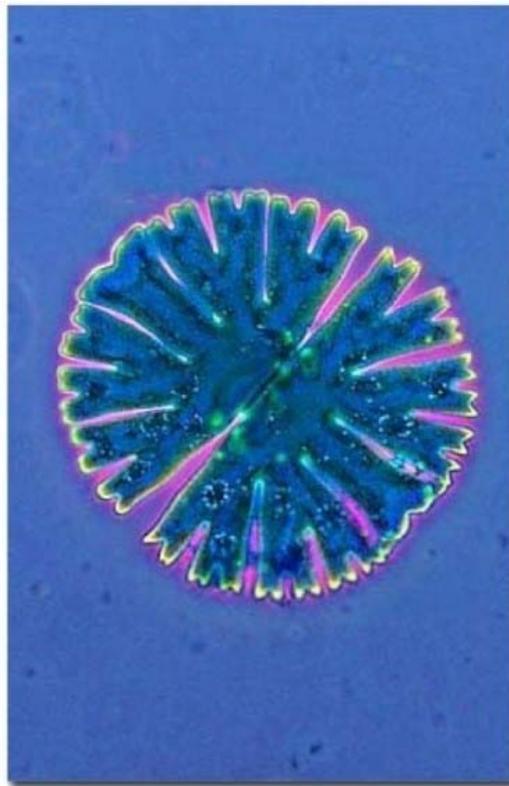


Figure 6: Phase contrast image of an unstained desmid (microscopic alga).

There are also a number of effects and artifacts that can modify the appearance of the image, in particular when the features under observation are near the resolution limit in size. For example, Fresnel fringe effects cause such features to reverse in contrast as the fine focus of the microscope is varied. A great deal of care must therefore be taken in interpreting the image, especially at higher magnifications, if useful information is to be obtained.

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### Biographical Sketch

**Michael Walls** was born in Liverpool and studied physics at the University of Sheffield before undertaking a Ph.D. at the Cavendish Laboratory in Cambridge. His thesis (1987) was based on theoretical and experimental studies in Electron Energy-Loss Spectroscopy (EELS) of surfaces and interfaces in the electron microscope. He then spent eighteen months performing post-doctoral research in the Laboratoire de Physique des Solides at the Université Paris-Sud, where he investigated using EELS the decomposition of carbonates under electron beams, and helped to develop algorithms for the elimination of EELS quantification errors due to fine structure in the spectrum. He returned to Cambridge in 1989 to conduct research on a range of materials using the scanning tunneling microscope (STM). In 1991 he joined his present laboratory, the Centre d'Etudes de Chimie Métallurgique in Vitry-sur-Seine where his research has included; the development of new microscopic techniques for of impurity element site determination using Energy-dispersive X-ray spectroscopy, the analysis in the transmission electron microscope (TEM) of the structure of metallic multilayers grown by electrochemical methods, the observation by atomic force microscopy (AFM) of corrosion processes in steels and of the formation of biofilms on metal surfaces, including steels and tantalum. He is also involved in the development of algorithms for quantitative analysis of AFM and STM and TEM images.