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Transmitted-light Microscopy for Biology: A Physicist's Point of View. Part 2

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5. Image contrast

Microscopical features can be resolved only if they are visible, i.e. if their images exhibit sufficient contrast. Contrast denotes how clearly the object is separated from its background. Usually it is expressed for brightness, but a colour difference is also suitable for this purpose. Techniques such as polarised light, interference microscopy and phase contrast microscopy are able to present variations in object thickness and index of refraction as colour differences. Colour contrast makes the observation of object details easier and more comfortable as compared to intensity differences. Where image contrast relies on intensity differences, it is assessed as

$$\text{Contrast} = |I_O - I_B| / I_B$$

Where $|I_O - I_B|$ is the absolute value of the difference between the respective luminous intensities of the object and its background, I_O and I_B, respectively.

The human eye can detect the existence of 2% contrast, but a brightness difference of about 5% is required under poor light conditions. For small objects the detection limit may increase further, up to 20% contrast (Bradbury & Evennett, 1996). These severe requirements can be avoided if visual observation is replaced by a CCD camera which provides contrast enhancement, either analogue or digital. A video-enhanced light microscope (VELM) is able to detect much smaller differences in intensity, thus detecting the presence of structures 5-50 times smaller than could be resolved with a conventional microscope, particularly if the VELM is combined with a reflection-contrast technique. It then permits objects as small as individual microtubuies (approximately 25nm diameter) to be studied in real time with high contrast, even though the objects observed may have their dimensions an order of magnitude smaller than the microscope resolution limit (Shotton, 1993; Ploem *et al.*, 1995). Another example of this electronic upgrading of light microscopy, known as 'nanovid' microscopy, is capable of detecting colloid gold particles having a diameter of 5-40nm and used to monitor cytoplasm dynamics (Geerts & Nuyens, 1991).

The sources of image contrast

Image contrast, and thus the appearance of the image, is not an inherent and invariable attribute of a microscopical object. Both contrast and image appearance derive from multiple light-specimen interactions, *including diffraction, absorption, refraction, reflection, light scattering, birefringence and. fluorescence* (Sanderson, 1994; Bradbury & Evennett, 1996). The observed image contrast depends critically on the respective role of particular light - matter interactions, which can be manipulated by the selection of the method of illumination and/or by the insertion of additional optical elements and filters between the objective and the eyepiece. Various methods of observation have been developed to enhance contrast, each suitable for the study of certain types of objects, or designed to bring out particular features.

Before the era of phase-contrast microscopy, objects in transmitted light microscopy were observed almost exclusively by their absorption of light. However, most living cells and thin tissue sections are virtually transparent and produce negligible intensity changes. Traditionally, the absorption of light by transparent objects has been enhanced by staining them with natural or synthetic dyes, staining reactions specific to certain cellular substances enabling chemically different parts of the cell to be discriminated by the hue or density of their colour. Staining techniques soon developed from a simple means of contrast enhancement into a powerful tool in histochemistry.

However, it is often necessary to inspect untreated material in either natural or controllable physiological conditions. Transparent objects in themselves change only the phase of transmitted light (hence they are known as *phase objects).* The object-induced phase shift is defined by the phase angle

$$\varphi = 2\pi/\lambda \,.\, [n.z]$$

where λ is the illumination wavelength and [n.z] the optical thickness of the specimen (n and z are its index of refraction and real thickness, respectively). To render phase objects visible the phase change must be converted to light intensity changes since neither the human eye nor a photographic film or electronic camera is sensitive to the phase properties of detected light. This goal has been attained with several microscopical techniques, particularly with *phase contrast, interference microscopy and differential interference contrast (DIC). Polarised light microscopy* might also be added to this category since its performance can be treated in terms of the detection of phase differences between two waves vibrating at right angles. Transparent objects may also be observed using their reflection, refraction and/or scattering of light. These effects generate contrast in *darkfield microscopy,* for many years the only microscopical technique capable of showing unstained phase objects. A more recent method that also exploits the effects of light reflection, refraction and scattering for imaging phase objects is *Hoffman modulation contrast (HMC).*

Image contrast and the back focal plane of the objective

The appearance of microscopical images can changed considerably by modulation of the diffraction pattern in the objective back focal plane. Each point in this plane contains wavelet contributions from all points of the specimen. Any mask or aperture (which we shall refer to here as a spatial filter) placed in the back focal plane of the objective will therefore affect the whole microscopical field simultaneously. Thus a properly designed spatial filter may serve as means of increasing contrast in the image. Oblique illumination techniques, including *dark-field microscopy, phase-contrast microscopy and Hoftman modulation contrast,* are based on this idea. On the other hand, spatial filtration plays no crucial role generating image contrast in such techniques *polarised light microscopy, interference microscopy* and *differential interference contrast.*

6. Transmitted light contrast techniques

In particular, a collection of transmitted light techniques is presented here to illustrate how manipulation of the light field in the objective back focal plane can influence the appearance of the image (oblique illumination, phase contrast and Hoffman modulation contrast). It is essential for these techniques that Köhler illumination be used, in order to provide defined and uniform obliquity of illumination in the object plane (for a nice survey of Köhler's method, see *Proceedings Roy. Microsc. Soc.* 28/4, 1993).

Phase contrast microscopy

Phase contrast was the first microscopical technique to provide researchers with the opportunity to obtain images of unstained living cells with sufficient contrast.[1] Its principle is represented by the scheme in Fig.6 (below).

FOOTNOTE [1] The phase contrast method was discovered while Frits Zernike was trying to assess the quality of a new concave grating purchased for his optical laboratory. When he took his first, and somewhat primitive, version of the phase contrast microscope to the Zeiss works in Jena (1932) he met much less enthusiasm than he expected. The influence of Abbe's inspiring personality had encouraged the attitude that everything worth knowing or trying in microscopy had already been achieved (Zernike, 1955).

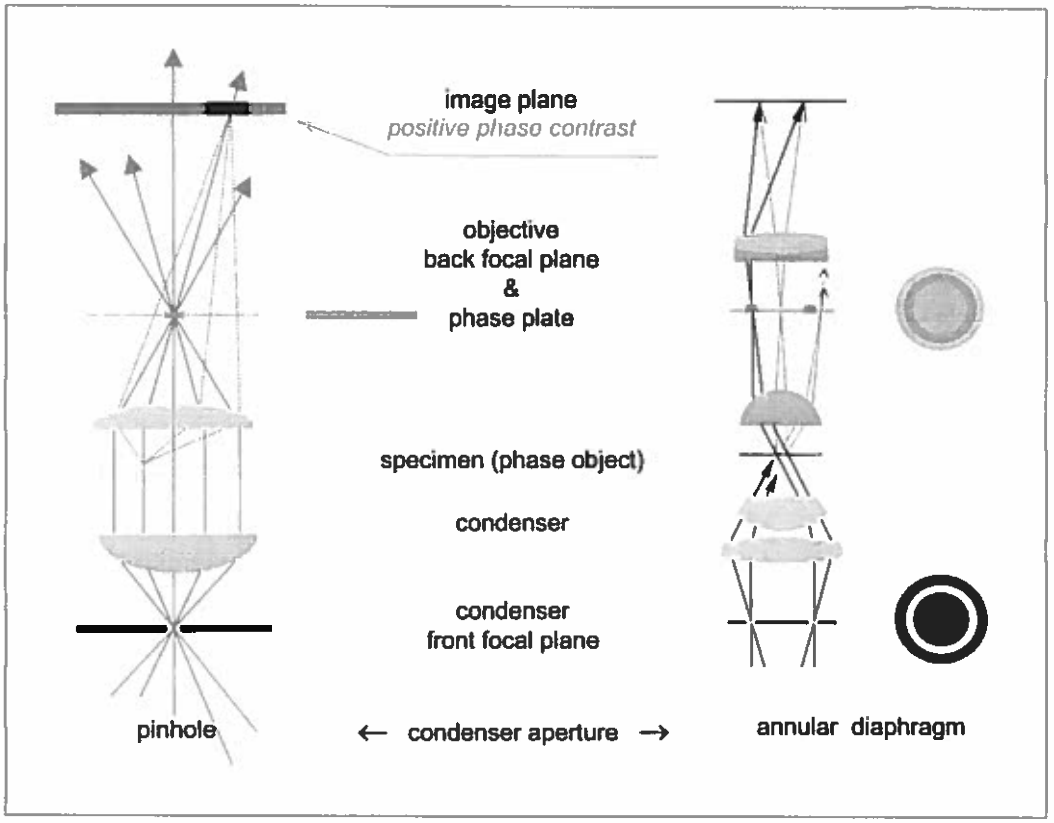


Fig. 6. Schematic diagram of a phase contrast microscope. Left side: the principle of a phase contrast microscope demonstrated for a collimated illumination beam. Right side: the common design of a phase contrast microscope, with an annular diaphragm and phase ring.

Here the specimen is illuminated with a collimated beam of light derived from a point source (i.e. a condenser pinhole) placed in the front focal plane of the condenser. All the direct (i.e. undeviated) light is brought to a focus in the objective back focal plane, where it forms an image of the light source. This image is arranged to fall on, and coincide with, a special region of a 'phase plate' made of a thin layer of a dielectric material, while the diffracted light (i.e. that which has been deviated from its original direction of propagation by its interaction with a specimen) passes through the remaining area. The aim is to introduce a defined phase shift (α between the direct and the diffracted light. Under this condition the object-induced phase shifts $\varphi(x,y)$ of the transmitted light are rendered visible by the interference of the direct light with the diffracted light.

This happens in the image plane where the associated pencils of diffracted and direct light that have emerged from each point in the object are again brought together (as shown in Fig. 1 in Part I of this article). To obtain an explicit relationship between the $\varphi(x,y)$ value and the resulting image brightness, $I(x'y')$, the effect of the phase plate must be examined using Fourier transform theory (Wolter, 1956; Born & Wolf, 1980). A typical phase plate designed for use with monochromatic green light retards or advances the waves of direct light a quarter of wavelength relative to the diffracted light by the phase angle ($\alpha = \pm \pi/2$ (i.e. $\pm 90°$). Then, for rigorous phase contrast (which means that both the condenser pinhole and phase plate are expected to be infinitesimally small) and for thin specimens that produce only small phase differences, the intensity in the image is given by a simple formula:

$$I(x'y') = C\,[T \pm 2\{\sqrt{T}\}\varphi(x, y)]$$

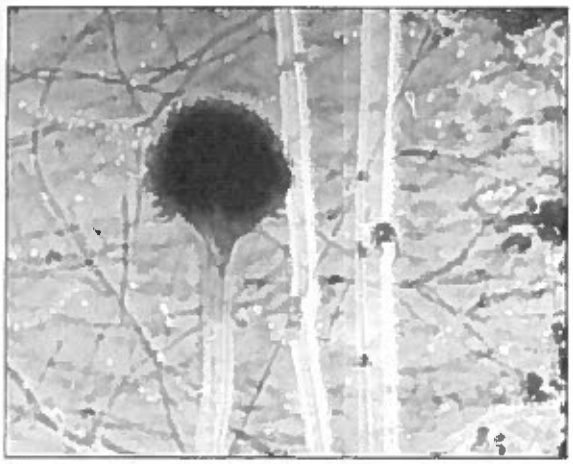


Fig. 7. *Aspergilus niger* mould in positive phase contrast. Scale bar (see Fig. 9) represents 100μm. From Plašek (1996).

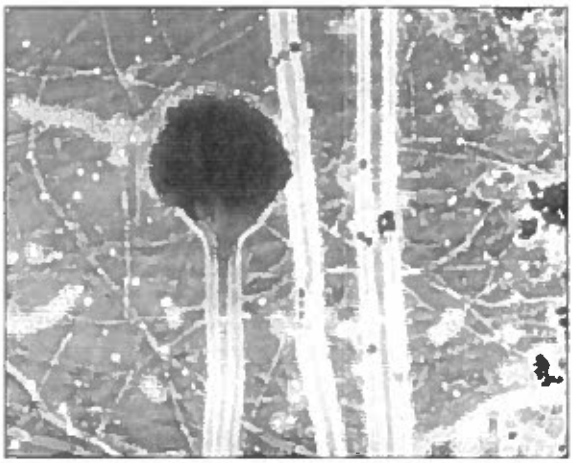


Fig. 8. *Aspergilus niger* mould in negative phase contrast. Scale bar (see Fig. 9) represents 100μm. From Plašek (1996).

Fig. 9. *Aspergilus niger* mould in bright field. Scale bar represents 100μm. From Plašek (1996).

where C is a constant, and T is the transmittance of the phase-changing region of the phase plate (Born & Wolf, 1980; see also Wolter, 1956). For a phase plate made in such a way that the optical path for the direct light is less than that for the diffracted light that passes around it ($\alpha = -\pi/2$), object features of greater optical path are seen as darker in intensity than their surroundings *(positive phase contrast)*. Using a phase plate that increases the optical path for direct light ($\alpha = +\pi/2$), the respective brightness of these object features and their background is reversed *(negative phase contrast)*, Figs. 7 and 8. With the explicit formula for I(x'y'), image contrast can be expressed as:

$$\text{Contrast} = |I_O - I_B| / I_B = 2\varphi\sqrt{T}$$

which indicates that the sensitivity of phase contrast can be increased if the phase-changing region is made partly absorbing ($T < 1$).

Phase contrast can also be performed in white light; small differences in brightness are then transformed into colour variations to which human eye is more sensitive (Pluta, 1989). In most actual phase-contrast microscopes an annular illuminating aperture is used, and the phase-changing region of the phase plate is ring-shaped, matching the image of an annular light source (Fig. 6, above). In this way, more illuminating light is obtained. Moreover, the loss in resolution, which would otherwise result from the small illuminating aperture typical of microscopes with pinhole illumination, is circumvented.

Unfortunately, phase contrast suffers from the halo bright light that surrounds some objects (for example the stalk image in Fig.7). This stems from the fact that in real instruments neither the condenser annulus nor the phase ring can be made arbitrarily narrow. A substantial part of the diffracted light can also pass through phase ring and undergo the same phase shift as the direct light. The halo is usually most pronounced at the edges of large objects whose diffracted light is concentrated in narrow cones at angles close to the direction of direct light (cf. Part 1, Fig. 4). This effect is also very disturbing in the images of highly refractive structures capable of deflecting pencils of direct light by either reflection or refraction such that they do not pass through the phase ring. Another ambiguity arises when $\varphi(x,y)$ is greater than $\pi/2$, and/or if the examined objects are stained. The effects of phase contrast and absorption may then combine in such way that image contrast is completely lost (see, example, the spores in Figs. 7, 8 and 9, left).

Interference microscopy and Nomarski differential interference contrast (DIC).

All interference microscopes are based on a fundamentally similar design: the light from a single source is split into two beams whose waves are coherent and in phase. One of them is made to pass through the specimen while the other is retained as a reference beam (Krug *et al.*, 1964). The reference beam of classical microinterference instruments is made to pass beside the specimen, sufficiently separated with respect to the size of the object. The two beams are then recombined. Their interference permits measurement of the optical thickness at any object point relative to its surroundings. Microinterference is a very valuable method that enables the refractive indices even the mass of tiny microscopic objects to be determined accurately, (Lacey, 1989; Dunn, 1998). Despite these merits, classic interference microscopy has practically disappeared from the laboratory. However, a new interference microscopy technique has been introduced that exploits local phase differences in transmitted or reflected light, rather than phase differences defined in relation to a single reference beam. Differential interference contrast, usually performed according to Nomarski (Fig. 10) is probably now utilised more than phase contrast for examining unstained transparent objects. It is especially well suited to observations of highly retractile objects (Padawer, 1968).

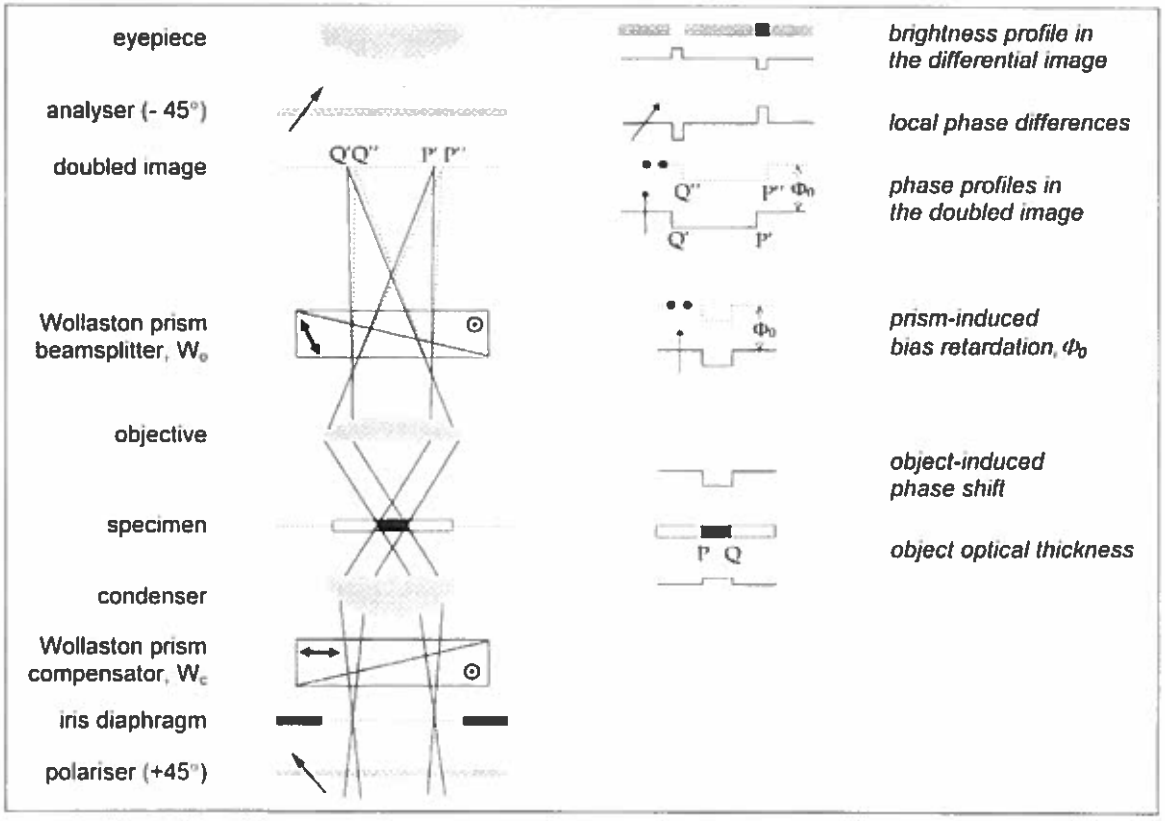


Fig. 10. Schematic diagram of a Nomarski DIC microscope. Left side: the microscope set-up. The illuminating light is first plane polarised by a polariser. Before reaching the specimen it passes a compensating Wollaston prism Wc. A second Wollaston prism (beam splitter Wo.) is placed close behind the back focal plane of the objective. The two perpendicularly polarised, divergent beams into which the image forming light has been split, are shown as full and dotted lines respectively, with their virtual common origins placed schematically into the objective back focal plane. The analyser defines in the overlapping images their matching parts of a given polarisation, and thus make them able to interfere. The directions of the optical axes in the two wedges of the prisms are indicated by the heavy pointed double arrows and by circles (axes perpendicular to the plane of the picture). This schematic diagram is based on the diagram presented in Padawer (1968) and seems to correspond to one of the first Nachet-Nomarski DIC microscopes. Note that the particular design of Wollaston prisms in other microscopes may differ from this scheme; we have failed to find detailed technical information on the construction of Wollaston prisms as used in some modern Nomarski instruments. Right side: illustration of phase shifts experienced by the image forming light on its passage from the specimen to the image, and the origin of the phase difference due to image shear. The azimuths of polarisation are indicated by arrows and dots.

Our presentation of the Nomarski DIC is based on papers by Nomarski and his collaborators (Allen *et* al., 1969) and Padawer (1968), who refers to personal consultations with the inventor of this technique. These two papers offer the interpretation of Nomarski DIC that may be considered as an educationally useful alternative to the usual approach found in microscopy textbooks. The Nomarski microscope differs radically from other microinterference instruments in the way in which two interfering light beams are obtained. Two Wollaston prisms are used for this purpose.[2] The objective prism (W_O) splits into divergent doublets all pencils of light that propagate from the specimen towards the eyepiece. As a result, the primary image is replicated into two overlapping images that are practically identical, except for their lateral shear and difference in polarisation. Thus two image points are obtained for each point in the object. Both the direction and amount of this shear are fixed by the beam splitter, the shear corresponding to a distance in the object less than the Rayleigh resolution limit (typically less than 0.2µm for 100x magnifying objectives). The image doubling cannot therefore be seen directly. However, its existence can be revealed by interference facilitated by the analyser, used to define parts in the overlapping images of an identical polarisation azimuth. Because the shear is very small, interference derives from local differences in optical paths, $\Delta(n.z) = (n.z)_A - (n.z)_B$, where subscripts A and B denote two adjacent points of an object that are separated at the shear distance. Those parts of the object where the optical thickness (n.z) is constant are revealed at background brightness, while the edges of objects exhibit either increased or decreased intensity relative to it, (Fig. 11), or in different colour. In this way, steep gradients of optical thickness in the object, e.g. such as defined by geometrical boundaries of cells and their organelles, are emphasised, and they appear in sharp contrast to their background. On the other hand, moderate slopes may generate little or no interference contrast even when they introduce quite large absolute optical path difference. Thus a Wollaston prism producing two replicated images and their shear of the magnitude comparable with the Rayleigh resolution limit acts as a spatial filter, enhancing high spatial frequencies and attenuating lower frequencies. Object details of the size approaching the Rayleigh resolution limit can therefore be imaged with a reasonable contrast. A tangible gain in a practical resolution of small details is thereby achieved (Allen *et al.*, 1969).

It is worthwhile to note that the Nomarski method makes it possible to use a large source of white light. Any extended source can obviously be considered as a collection of point sources that contribute to the resultant interference image. Moreover, since the source image is projected into the objective back focal plane, such individual contributions would exhibit undesirable additional phase shifts between their perpendicularly polarised components induced by the beam splitter W_O itself, and their respective magnitudes vary with the place where the light has actually passed the sandwiched wedges made of birefringent quartz. This would normally result in a loss of interference contrast in the image. To avoid this, a compensating Wollaston prism (W_C) is fitted in the first focal plane of the condenser, designed to produce an additional phase shift, combined with the effect of the Wollaston prism behind the objective so as to provide a constant value for all source points.[3] By the same token this bias retardation is added to all interfering light waves in the image plane. Its magnitude, Φ_O, and hence image contrast, can be controlled by a slight lateral displacement of the entire beam splitter.

FOOTNOTE 2 A Wollaston prism is a birefringent device that splits a plane-polarised beam of light into two divergent beams that are plane polarised at right angles and emerge from the splitter symmetrically with respect to its optical axis if the incident light is parallel to this axis. Nomarski employed a modified Wollaston prism, cut in such a way that the pairs of related beams diverge at a very small angle of about 1 arc minute or even less, and intersect in a virtual origin that occurs outside the splitter. Hence the apparent origin of the divergent pairs of beams produced by the W_O splitter can easily be placed in the back focal plane of the objective, which is usually located somewhere between the lens elements.

FOOTNOTE 3 In microscopy textbooks the respective roles of Wollaston prisms Wc and W_O are usually presented as a beam-splitter and beam-combiner. In our opinion, such an approach is less clear than Nomarski's original explanation of how this particular DIC instrument acts.

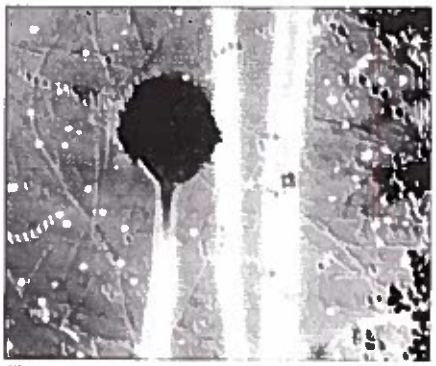


Fig. 11. *Aspergilus niger* mould in Nomarski DIC. Scale (as see Fig. 4) represents 100µm. From Pluta (1996).

DIC images of transparent objects give an impression of a relief as if the object is illuminated from one side. Edges thus have either increased or decreased intensty relative to a grey background, and tend to be coloured in interference colours if white light illumination is used. This colour contrast can also be controlled by displacing the beam splitter laterally, thus adjusting the magnitude of bias retardation. In practice, this retardation is adjusted to produce the optimum shadow-cast effect. The sensitivity of DIC extremely high, allowing us to detect an optical path difference of about 2nm (Padawer, 1968). Moreover the shadow-cast images are free of haloes as seen in the phase contrast. Another commonly acclaimed feature of Nomarski DIC is its ability to display images with extremely shallow depth of field (Inoué, 1986 However, this is actually only pseudo-sectioning, since all the defocused regions of the object still appear in the image (Cogswell & Sheppard, 1992).

The pseudo-sectioning effect stems from the relationship between the rate of defocus blurring and the size of image features: very small features, which appear excessively prominent in images obtained with edge-detection techniques such as DIC and Hoffman modulation contrast, are blurred earlier than the large ones. In particular, small object details lying more than about three Airy disc radii above or below the plane of focus do not materially contribute to the generation of contrast and disappear within a background (i.e. the contrast of blurred small details in the overall image turns out to be comparable with the low contrast images of gentle optical path gradients that are often typical for large parent objects), (Allen *et al*, 1969). A considerable disadvantage of DIC is that the microscope is basically a polarisation instrument and therefore acts simultaneously as a polarised-light microscope. Any birefringent material in the specimen is thus betrayed by its bright appearance certain orientations. DIC is therefore not suitable imaging cells grown on birefringent plastic supports.

Dark Field & Oblique illumination

Fig. 12. *Mucor mucedo* black bread mould in dark field. Field size is 625 × 440µm.

Dark field microscopy was the first widely accepted technique to exploit anaxial illumination as a regular tool for the visualisation of transparent objects. The condenser is modified so that the illuminating light cannot enter the objective unless it is scattered, diffracted or reflected by an object (Bradbury & Evennett, 1996). The background to the field of view is thus black, with objects appearing bright. Note that from a formal point of view this technique is equivalent to the phase contrast performed with a non-transparent 'phase plate' ($T = 0$). Dark field microscopy is very useful in scanning a field for tiny unstained objects, particularly if their dimensions are below the Rayleigh resolution limit (ultramicroscopy). Unfortunately, this property of dark field microscopy may be disturbing in studies of large and complex objects, where small details become too prominent while the large weakly refractive structural feature that are needed for most informative images are usually not visible (Fig. 12).

This disadvantage of dark field microscopy can be overcome if the oblique illumination is arranged to allow some direct light to contribute to the image formation. It is a paradox that this possibility has been known since 1869, when Ernst Abbe invented his excentric substage and demonstrated clearly the capability of oblique illumination to increase both the resolution and contrast in the microscope image (Bradbury, 1996). This property of oblique illumination was however not fully exploited until the introduction of modern methods such as the various modes *of single-sideband microscopy* (Kachar, 1985; Bretschneider & Tennis, 1994, and also references therein), *Hoffman modulation contrast microscopy* (Hoffman & Gross, 1975), and the *direct-view 3-D microscope* of Greenberg and Boyde (1997).

As already mentioned, oblique illumination may provide super-resolution. Nevertheless, the asymmetrical expansion of the acceptance cone of the objective is not the true equivalent of a high-NA objective. Note that while diffracted light from one side of the diffraction pattern is allowed to enter the objective at an expanded acceptance angle, its counterpart from the opposite side is partly rejected. Super-resolution is obtained at the expense of image contrast (Figs. 1 and 2 in Part I of this article). To improve contrast, the intensity of the direct light must be partly attenuated (Bretschneider & Tennis, 1994). The performance of both single-sideband and Hoffman modulation contrast (HMC) microscopy is based on a selective spatial filtering of the diffraction pattern in the objective back focal plane. We will illustrate this approach with reference to the latter technique.

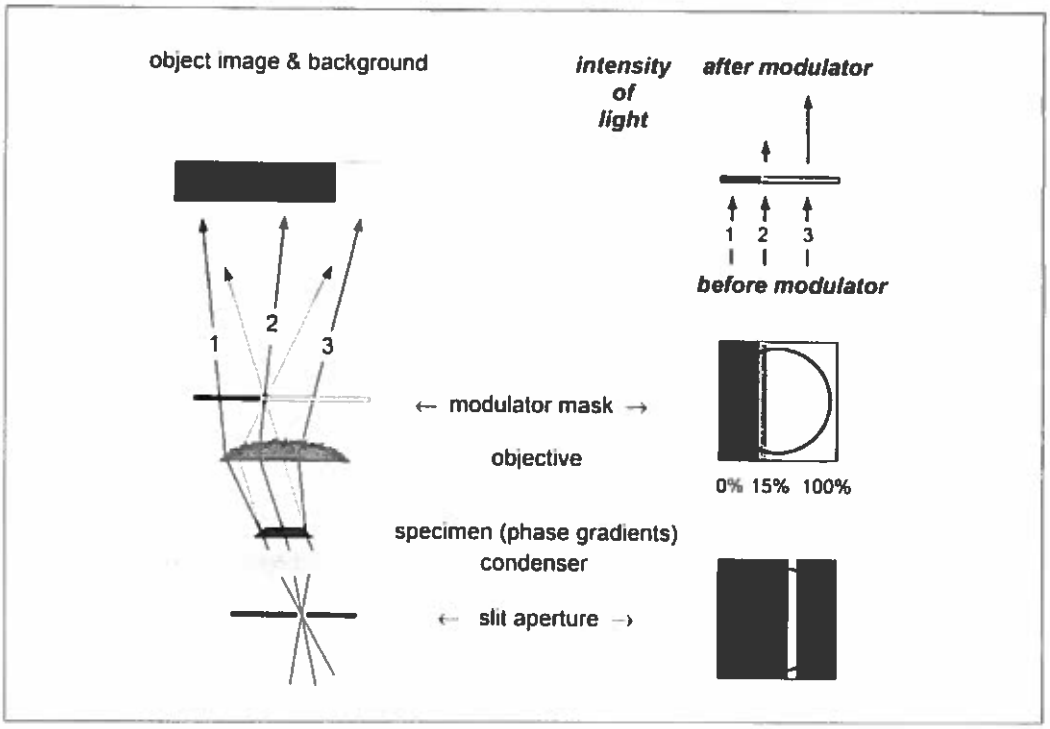


Fig. 13. Schematic diagram of a Hoffman modulation contrast microscope. Full and dotted lines represent respectively the illumination light deflected by phase gradients and its ideal path without a specimen.

The HMC system uses a slit aperture located in the front focal plane of the condenser and a spatial filter (modulator) consisting of three zones, in the back focal plane of the objective lens (Fig.13). If the slit aperture is moved to one side of the front focal plane of the condenser, the illumination is oblique and super-resolution is obtained. The modulator must be aligned in such a way that the luminous image of the slit falls on and coincides with its grey region. Then it attenuates the intensity of direct light and thus increases the contrast of very small image features. Moreover, the modulator converts gradients of optical thickness (n.z) into intensity variations in the image, thereby also making large object details more clearly visible. An optical gradient behaves like a miniature prism inside the specimen (Wolter, 1956), deflecting the direct light. Depending on the direction of this deflection, the direct light will fall on the region of either maximum or minimum transmittance of the modulator. The corresponding image of the 'prism' will then appear either darker or brighter than the object background. Since the effect of increasing optical thickness is opposite to that of decreasing optical thickness, the modulator produces shadow-cast images, very similar to those obtained with Nomarski DIC. As compared with Nomarski DIC microscopy, HMC may be less efficient in imaging very tiny phase objects (*cf.* the images of mycelium in Figs. 11 and 14). On the other hand, the images obtained with HMC are usually less obscured by the effects of out-of-focus phase objects. Moreover, HMC microscopy is insensitive to birefringence in the specimen or its support.

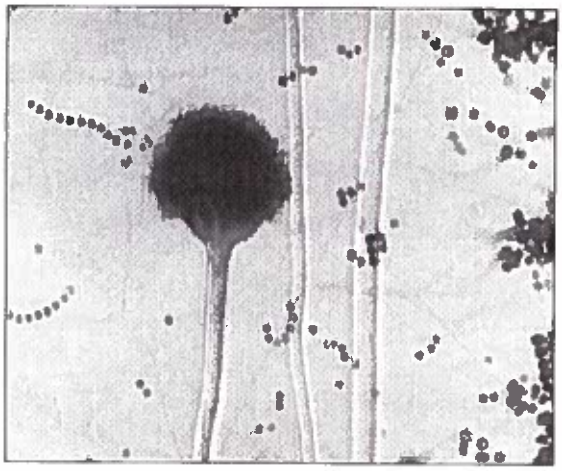


Figure 14. Aspergilus niger mould in Hoffman Modulation Contrast microscope. Scale bar (see Fig. 9) = 100 µm.

No particular spatial filter is used in the direct-view 3-D microscope since, depending on the obliquity of the illumination, the direct light may be stopped partly by the objective aperture itself. Advanced direct-view 3-Dmicroscopes utilize simultaneously four oblique illumination beams, positioned at 90-degree steps from one to another. This leads to improved resolution throughout the entire image plane instead of the partial improvement in one preferred direction that is typical for a standard single anaxial illumination (Greenberg & Boyde, 1997). However, the main benefit of employing multiple oblique illumination is the possibility of arranging the four illuminating beam as left and right pairs to give rise to parallax between the right and left views of the blurred out-of-focus images. Using polarising filters it is possible to encode and subsequently separate these left- and right-eye views. A 3-D image of the object can then be seen directly through the microscope, exhibiting the sharp image of a focused plane with the blurred out-of-focus images clearly separated from it along vertical axis.

Polarised light microscopy

Fig. 15. *Mucor mucedo* black bread mould in polarised light microscopy. Field size is 625 × 440µm.

Apart from the need for strain-free lenses for special instruments, polarised light microscopy can be performed with any transmitted light microscope fitted with a polariser in its illuminating light path and a analyser in the imaging light path, crossed in relation one to the other. Therefore no light can reach the eyepiece unless a birefringent object is placed under the microscope. If properly oriented, the birefringent material splits the plane-polarised illumination light into two waves vibrating at right angles. Part of the light can then reach the eyepiece, and the birefringent object is seen on a dark background often in vivid interference colours that are determined by the specimen thickness, its orientation, and degree of birefringence (Hartshorne & Stuart, 1970).

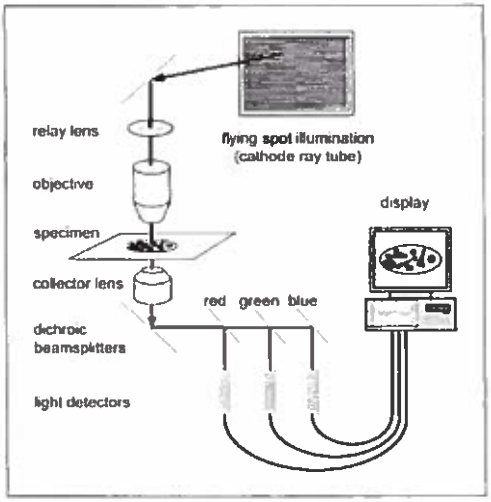
Another important component of the polarised light microscope is a retardation plate - a birefringent device inserted between the crossed polars, designed as e.g. a λ/4 plate for green or yellow light (λ from 550 to 575nm). It adds a defined birefringence to the whole microscope field, which then lights up in a brilliant interference colour. Hence a bright image is obtained even for the tiny birefringent objects often found in biological specimens (Fig. 15). This image includes also the non-birefringent features of the object, superimposed in the form of a bright-field image. By rotating the retardation plate, the image colours can be tuned to optimal contrast; this is not necessarily achieved at the 45° orientation of the retardation plate, which is the standard set-up for mineralogical studies. This simple and quite general physical phenomenon has unfortunately been misrepresented as a universal tool for tissue specific studies of living microorganisms (Ho & Lawrence, 1993). Intrinsic birefringence is a characteristic of various specimens, ranging from crystals other than cubic to regularly arrayed macromolecules or submicroscopic structures, e.g. cellulose fibres, bone sections, striated muscle or regularly arranged pseudopodia of moving protozoans. Moreover, tiny elongated objects can be visualised with the polarised light microscope even if their material exhibits no real birefringence, owing simply to the directional properties of light scattering by elongated objects (Arimoto & Murray, 1996). The biological applications of polarised light microscopy practically ceased following the introduction of phase contrast microscopy and the enormous productivity of electron microscopical investigations of fixed cells. However, there are some indications that the method has

withstood the test of time and can still provide information complementary to other analytical methods in biology (Whittaker, 1995)

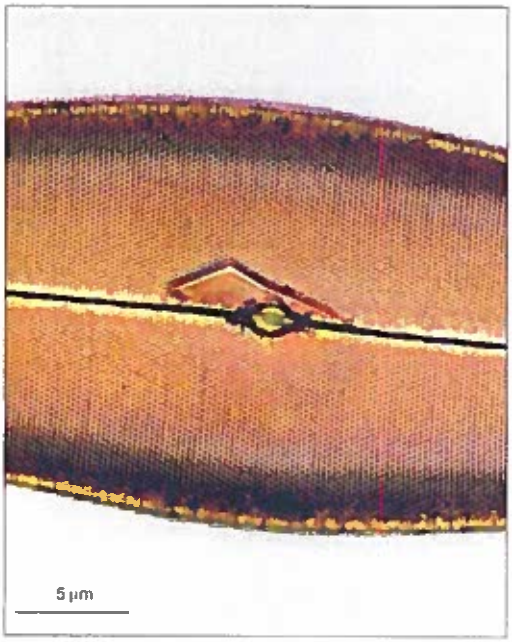
Flying spot microscopy

Quite recently, a non-confocal transmitted-light scanning microscopy named COSMIC (for COlour Scanning MICroscope) was introduced. It is based on the flying spot technique developed in the 1950s (Roberts & Young, 195 1; Gravely *et al.*, 1994). The primary light source is a cathode ray tube that produces a white raster pattern on its screen (Fig. 16).

Fig. 16. Schematic diagram of a *COSMIC* Flying Spot Microscope.



A moving light spot is projected by a relay lens and the microscope objective on to the sample, where it forms an illuminating spot of Airy disc diameter. The light transmitted through the specimen is collected by a condenser lens and then divided into red, green and blue (RGB) detector channels. The full colour image of the object is reconstructed using the x-y positions of the scanning spot and three corresponding digitised colour intensities, at frame rates fast enough to examine live samples (12Hz). This scanning system is capable of a unique contrast enhancement via the modulation of the illumination spot brightness in inverse proportion to the absorbance of the sample.



COSMIC oversamples the image beyond the common Nyquist condition (Inoué, 1986), taking about 3.5 electronic data points per optical resolution element. This is beneficial to imaging objects that possess big differences in the transmittance and/or optical thickness of adjacent structural details, such as the puncta of diatoms, and their walls. For example, with a 40x/NA0.75 objective, the Rayleigh resolution limit of which is 0.45µm, the puncta of the diatom *Pleurosigma angulatum* (which have a diameter of approximately 0.25µm) can be recognised much more clearly than with normal bright field microscopy (Fig. 17). The excellent image contrast achieved with the COSMIC is obviously facilitated by the fact that point illumination is used From this point of view it is equivalent to the use of a very small illuminated field diaphragm, known to increase dramatically the image contrast in a standard transmitted light microscopy. COSMIC seems to be a very promising technique, but it needs further testing.

Fig. 17. A diatom, *Pleurosigma angulatum*, seen with the COSMIC microscope (40 /0.75 Zeiss Plan-Neofluar objective). Scale bar represents 5µm. This picture was kindly provided by Dr Ben Gravely.

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