Reference: *Proceedings of the Royal Microscopical Society* June 1998 33/2: 121-127

Transmitted-light Microscopy for Biology: A Physicist's Point of View. Part 1

JAROMIR PLASEK AND JOSEF REISCHIG*

Institute of Physics of the Charles Univervity, Ke Karlovu 5, 121 16 Prague, and
**Faculty of Medicine, Charles University in Pilsen, Karlovarská 48, 301 66 Pilsen, Czech Republic.*

1. Introduction

Why might it be important to pay much attention to physical theories if everybody knows that, for example, a proper handling of examined specimens is without any doubt a crucial step towards getting good results? However, when microscopy is to be used as a tool for solving complex problems, microscopists cannot rely upon their routine knowledge of a single technique; they must be familiar with a wide range of microscopical modes and techniques, and be able to evaluate and then interpret the results so that the true significance of their findings can be relayed to other co-operating experts. This goal cannot be achieved without an understanding of the physical principles that underlie their everyday practice. The renaissance of light microscopy over the last 20 years might be another good reason to refresh occasionally the learning of physical principles of new microscopical techniques.

We shall concentrate on transmitted light microscopy, with special emphasis on its use in biological research. For practical reasons we cannot go into much detail on recent innovations of computerised microscopy, which concerns both the powerful scanning techniques (such as confocal microscopy, scanning near-field microscopy or two-photon microscopy) and the data processing of digitized images that can be grabbed at high sensitivity with sensitive charge-coupled devices (CCD cameras). Instead, we will discuss in some depth those aspects of light microscopy that are directly related to the interaction of light with material objects. In particular, we will analyse the main sources of image contrast, including the interplay of the light - matter interactions and the optical effects of particular microscopical techniques. Our aim is to outline modern views of image formation without their usual rather mathematical treatment, and to make them comprehensible even for those readers who do not have a sufficient background in mathematics. Nevertheless, we assume that these readers are familiar with at least the basic concepts of wave optics, such as wavelength, the velocity of light and its dependence on the index of refraction, phase of harmonic wave, coherence of light, interference, diffraction and birefringence.

2. Indispensable basics

In a microscope we are able to see *detail* in specimens too small to be visible to the naked eye. This is made possible by two basic elements of the microscope optics - the *objective* and the *eyepiece*. These lens systems together define the overall *magnification* of the microscope, while its *resolving power* derives mainly from the quality of the objective. In a classical compound light microscope the objective collects light emanating from the specimen and focuses it to form a magnified image. The eyepiece merely magnifies this image further, to adjust its resolved details to the resolving power of either the human eye, or some recording device, such as photographic film or a CCD camera. The objective is clearly the most important component of any microscope. As a result, the role of the *condenser* has often been undervalued. No doubt its main function is to obtain a sufficient intensity of illuminating light, but it also affects the resolving power and usually plays a decisive part in generating image contrast.

Classical microscopes display the entire field of view at one time. An alternative to this scheme is a scanning microscope. In scanning techniques such as *confocal microscopy* (Pawley, 1995) or lensless *scanning near-field optical microscopy* (Cherry, 1991), the image is generated by serial recording of individual picture elements, or *pixels*. The serial build-up of the image makes it possible to improve the resolving power of light microscopes over the limiting value of conventional compound instruments. Unfortunately any serial method means

computer-aided processing of large sets of pixels, and this may be relatively slow. Insofar as we are interested in instruments using lenses, we may conclude that in both classical and scanning light microscopy the resolution limit is determined by the quality of the objective lenses. The design of microscope optics is a very specialised field that made great progress due to the use of powerful computers and to advances in glass technology. Microscopes now provide images with higher contrast and of much higher quality than ever before (Kapitza, 1996). No attempt will be made to cover this subject here.

3. Diffraction limits of resolution

Fundamental limits of microscopic resolution (i.e. the capacity to distinguish and separate details in a specimen) are related to the wave properties of light. The wave theory, particularly its Huygens-Fresnel principle, has been employed in the interpretation of microscopical resolution in two alternative ways:

(i) in the theory of light diffraction by circular apertures of objectives, and
(ii) in Abbe's theory of the microscope, including its modern upgrading based on *Fourier optics.*

Rayleigh resolution limit

Because of diffraction, no objective can produce a sharp point-like image even when the light that has emerged from a point object is accurately focused. Instead, a three-dimensional diffraction pattern is formed, the size of which increases with decreasing the objective aperture. The projection of this diffraction body on to the image plane appears as the well-known Airy disc pattern, the size of which defines the ability of the microscope to separate images of two adjacent object points. Usually the Rayleigh criterion is used to assess a theoretical resolving power of the microscope. For incoherent light (emitted by, for example, self-luminous fluorescing objects) it leads to the equation

$$\delta = 0.61\ \lambda / NA_{obj}$$

where δ is the minimum distance between two just resolvable details of the object, and λ is the wavelength of light in vacuum. NA_{obj} , the numerical aperture of the objective, represents the angle included by a cone of accepted light. $NA = n.\sin\alpha$, where n is the refractive index of the medium between the objective lens and the object, and α is the angle between the optical axis and the most oblique ray entering the objective and contributing to the image. For coherent illumination the overall light intensity in overlapping Airy discs is subject to interference phenomena. In this case the estimation of resolving power is moderately worse than for the incoherent illumination, with the numerical factor equal to 0.77 instead of 0.61 (Born & Wolf, 1980).

In real microscopes the actual degree of coherence of the illumination light is determined by the condenser aperture. This important fact has seldom been noted in textbooks, nor has its consequence been discussed accordingly. The impact of the numerical aperture of the condenser, NA_{cond} on the resolution of point objects was considered by Hopkins & Barham (1950). Their results suggest that resolving power is optimal for $NA_{cond} \approx 1.5 \cdot NA_{obj}$ under which condition it should be about 10% better than for the incoherent illumination. The common rule, $\delta = \lambda / (NA_{obj} + NA_{cond})$, does not strictly match these findings. It also leads to an absurd result when NA_{obj} tends to zero, unless qualified by the condition $NA_{obj} \geq NA_{cond}$. Note that residual lens aberrations (and sometimes also the lack of proper skills in using the microscope) may lead to a practical resolution far below the theoretical value. In practice one usually has to compromise between the resolution and the image contrast. Experience shows that a ratio of NA_{cond} to NA_{obj} of about $2/3^{rds}$ is the best choice for bright-field microscopy (Pluta, 1988), or $4/5^{ths}$, athough this rule of thumb can be broken when image contrast is boosted electronically (Inoué, 1986).

It is obvious that microscope resolution can be improved by either increasing the objective numerical aperture or decreasing the wavelength of light. The latter fact led very early to the development of ultraviolet microscopy (by Köhler in 1904), usually carried out in bright-field at wavelengths between 280 and 400nm. Apart from the improved resolution, the use of UV may enhance contrast in biological specimens owing to its absorption by

proteins and nucleic acids. Nevertheless it is in most cases a method auxiliary to other techniques rather than the only means of investigation, since the UV may cause severe damage to living cells (Pauluzzi *et al.*, 1996).

Beyond the Rayleigh criterion

The Rayleigh criterion is appropriate only for the visual observation of images when the minimum detectable dip in the midpoint of overlapping Airy discs is about 26.5% of the maximum intensity. Therefore the Rayleigh resolution limit does not exactly hold true for techniques that rely on the electronic processing of images, such as confocal microscopy and video-enhanced contrast microscopy. The lateral resolution of fluorescence confocal microscopy is improved by a factor of 1.4 compared to the Rayleigh resolution limit of classical microscopy (Pawley, 1995). A similar improvement in the resolution limit can be obtained using video-enhanced contrast microscopy, which allows one to reduce the required dip between two overlapping Airy discs to about 2 or 4% of the maximum intensity (Inoué, 1986). However, such contrast-enhanced images must be interpreted with great care and caution because the apparent maxima in two overlapping Airy discs will be found to be closer than the true midpoints of the individual contributions, whenever the distance of these true midpoints is less than the Rayleigh limit. This is a very confusing effect that may lead to a false overestimate of resolution improvement. However, all these improvements in lateral resolution are usually of little practical importance. Confocal microscopy is noted mainly for its unique optical sectioning property, while the novelty of contrast-enhanced microscopy consists of its capability to see objects that may have their dimensions an order of magnitude smaller than the Rayleigh limit (Shotton, 1993).

Microscopical resolution can also be improved beyond the Rayleigh resolution limit of $\delta = 0.61\lambda/NA$ by oblique illumination, i.e. under the condition when super-resolution has been observed (see below). Such super-resolution is normally interpreted within the framework of Fourier optics theory (Rhodes, 1953; Kachar, 1985). To explain it in a way that would be compatible with the preceding scheme of overlapping diffraction patterns we have to take into account the actual brightness distribution. A 'point', the image of which is the common Airy disc, is a mathematical abstraction sanctioned for self-luminous objects only. In reality, the diffraction patterns corresponding to the smallest resolvable details of real objects may exhibit very different brightness profiles, dependent on the form of a particular object detail, its index of refraction and the obliquity of illumination. Super-resolution will take place if these brightness profiles are steeper than the central peak of the Airy disc. Super-resolution exists usually in concert with contrast enhancement that obviously facilitates the visibility and recognition of small object features and thus full utilisation of the super-resolution effect.

Depth of field

Considering the axial dimension of the three-dimensional diffraction body we can define a vertical resolving power of objective, in a similar way to lateral resolution. For bright-field microscopy with high-aperture objectives, this vertical resolution approximates to the depth of field. Considering different resolution criteria and approximations, a number of different expressions for depth of field can be found in the literature (Inoué, 1986; Lacey, 1989; Young, 1989). According to Françon (1961), the diffraction limited estimation of the depth of field, Δz, is given as:

$$\Delta z = \lambda . n / [4.NA^2_{obj}]$$

Depth of field represents the distance along the optical axis throughout which an object appears to be sharply in focus. The above formula indicates that a high microscopical resolution must always be obtained at the expense of very small depth of field. On the other hand, shallow depth of field gives rise to the optical sectioning ability of the microscope. In classical microscopes a focused image is often degraded by light from out-of-focus object planes. A significant improvement in both image contrast and sectioning can be achieved only by reducing this stray light, by either confocal microscopy or a computational procedure, known as image restoration (Pawley, 1995).

4. Modern views of image formation

The back focal plane of the objective and Abbe's theory of image formation

Abbe elaborated his theory using a diffraction grating as a model microscopical object (Evennett, 1996). When light passes through such a grating, it is spread out by diffraction. With an illuminating cone so narrow that it is nearly like a collimated beam of parallel rays (as Abbe assumed to simplify the mathematical treatment of the diffraction problem), the spatial distribution of the intensity of light behind the grating *(the diffraction pattern)* will exhibit distinct maxima. These maxima are best resolved in the back focal plane of the objective, following a rule of geometrical optics according to which all parallel rays entering a positive lens are brought to a focus at a distinct point in its back focal plane, Fig.1.

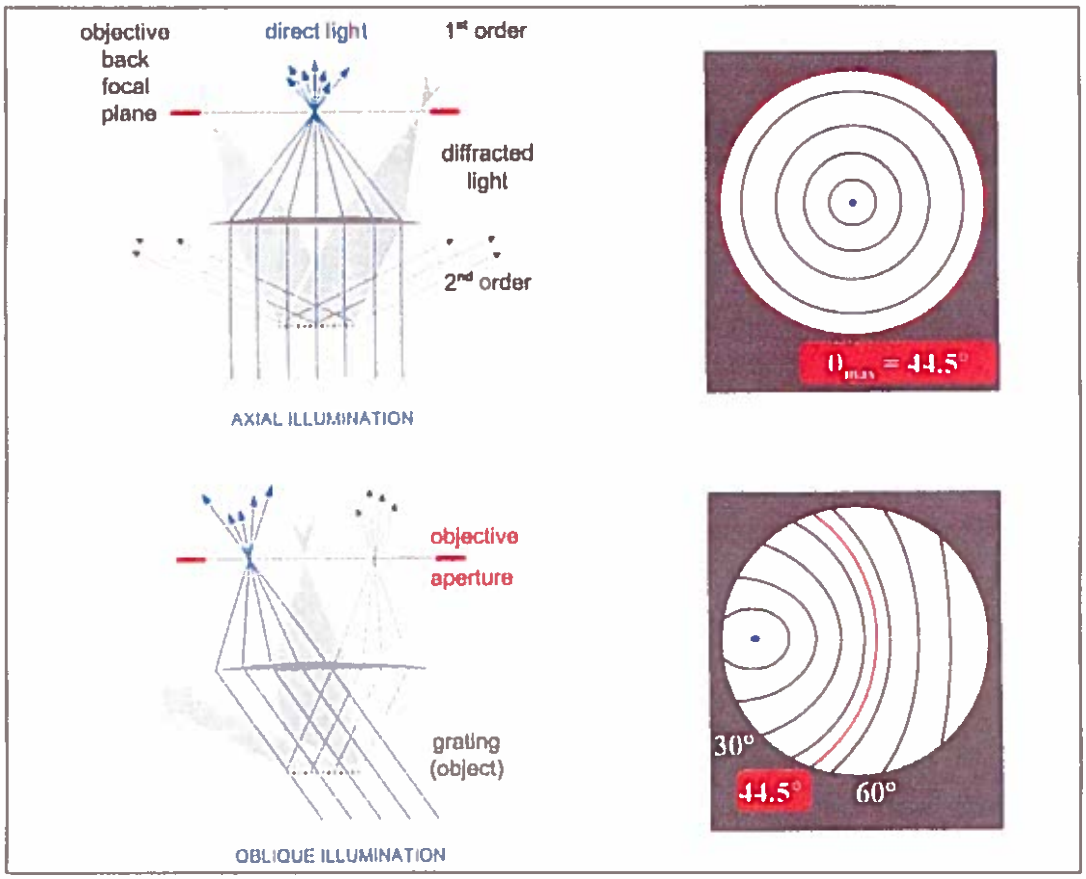


Fig. 1. Objective back focal plane. Left side: relationship between the direction of direct and diffracted light and the position of their respective foci in the objective back focal plane (the oblique illumination beam is tilted from the optical axis of the microscope by the angle of 35°, and the numerical aperture of the lens is NA = 0.7). Right side: top view of the objective aperture. The bold dot represents the focus of the direct light, and full lines mark the foci of light diffracted at defined angles, increasing in 10° steps. The red lines mark the maximum acceptance angle of a *NA* = 0.7 objective. The diagram shown at bottom right illustrates the expansion of the acceptance angle typical for oblique illumination.

The most intense of them *(zero order diffraction maximum)* is not deviated from the direction of the original light beam. The essence of Abbe's theory consists in a conclusion that the real periodicity of the grating can be revealed only if both the zero and first order diffraction maximum at least is not occluded by the objective aperture and thus is allowed to contribute to the image. Any additional higher orders of diffracted light (i.e. the light that has been deflected more widely from its original direction of propagation) will enhance the fidelity of the grating image (Fig.2). On the other hand, if only these higher orders of diffracted light are available, a spurious image may be formed. For example, without the proper contribution of the first order, the image of the grating will exhibit false spatial structure of double periodicity (Born & Wolf, 1980). Using a diffraction formula that relates the spatial separation of the neighbouring diffraction maxima to both the grating dimensions and the wavelength of light, Abbe also established a relationship between the resolving power of the microscope, objective aperture and wavelength, which is practically identical to the Rayleigh limit.

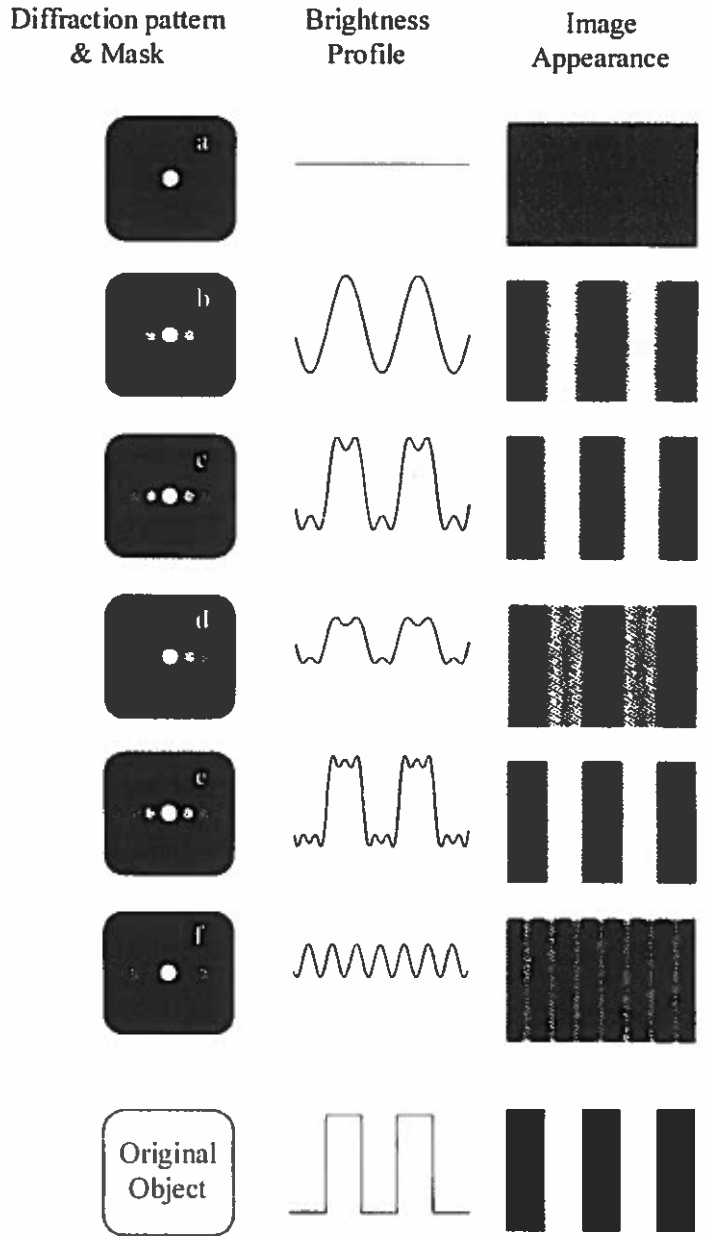


Fig.2. Diffraction grating images to illustrate Abbe's theory: a computer simulation. Fourier transforms of a diffraction grating (bottom right) were calculated. Then some diffraction spots were selectively filtered and the remaining ones used to calculate the inverse Fourier transforms i.e. the resultant images of the grating. Left column: the diffraction maxima used to calculate the inverse Fourier transforms: (a) direct light only, (b) direct light and first-order maxima, (c) direct light and the first- and second-order diffraction maxima, (d) direct light and the first- and second-order diffraction maxima with one half of the diffraction pattern rejected, (e) direct light and the first-, second- and third-order diffraction maxima, (f) direct light and the first- and third-order diffraction maxima, without the second-order. Central column: brightness profiles in the calculated grating images. Right column: the appearance of the calculated grating images. The data used to draw this figure were kindly provided by Professor Jiri Bok, Institute of Physics, the Charles University, Prague.

Fourier optics approach

Abbe's theory started a revolution in the design and performance of light microscopes. Despite this, for many years it was firmly rejected by most practical microscopists of his time who said that this theory showed only how the microscope might be used by physicists for interference experiments (Zernike, 1955). No doubt the extrapolation of the grating image studies to biology may still appear to be a confusing issue since a typical biological object is aperiodic and thus does not form a regular grating-like diffraction pattern that is easy to understand. Diatom frustules, the beloved object of the nineteenth century microscopists, are just a rare exception to this rule. An appropriate upgrading of Abbe's theory has been provided by the Fourier optics theory of image formation. This exploits a mathematical theorem according to which any function in space can be broken down into an infinite set of undulating sine and cosine components of various frequencies, the amplitudes and phases of which are encoded as the function of frequency that is called Fourier transform. Unfortunately, this represents a rather mathematical treatment which might be inappropriate for many practical microscopists (Franqon, 1961; Martin, 1966; Castleman, 1979; Goodman, 1988).

A link between the process of image formation and Fourier transforms stems from the Huygens-Fresnel principle. According to this, each point along a propagating wavefront may be considered to be the centre of a secondary disturbance that leads to a spherical wavelet. It says that the wavefront at any later instant may be regarded as the result of the mutual interference of all the wavelets emanating from the preceding wavefront. In case of light transmitted through material objects, both the amplitudes and relative phases of the wavelets are affected by the optical properties of the object, such as its absorbance, index of refraction and thickness. To describe how the local intensity and/or phase of the transmitted light varies across the field of view, a special function is introduced that is *called object transmission function, F(x,y);* here *x,y* denote co-ordinates within the object plane. The wavefront that has been changed by interaction with the object propagates further towards the objective. It passes the objective back focal plane and then reaches the primary image plane where an image of the object is formed (Fig. 3). We will initially assume that the specimen is illuminated with a collimated beam of monochromatic light, i.e. with a plane wave. In these circumstances the wavefront observed in the back focal plane of the objective exhibits a remarkable property: it is equal to the Fourier transform of the transmission function F(*x,y*) of the whole object plane (i.e. of the object itself and its background).

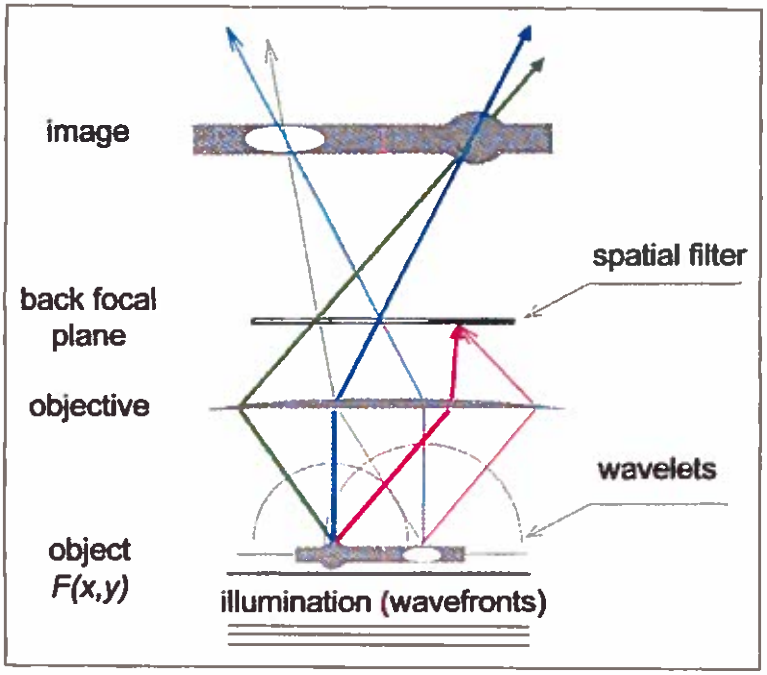


Fig.3. Illustration of the Huygens-Fresnel principle which leads to the formation of Fourier diffraction patterns in the back focal plane of the objective, and the idea of spatial filtration of the wavefronts in the objective back focal plane. The direction of propagation of diffracted light is coded by colours, the arrow thickness marks the intensity of the transmitted light, and the wavelet radius indicates the phase of the transmitted light.

Furthermore, the primary image of the object can be interpreted in terms of a reverse Fourier transform of the wavefront formed at the objective back focal plane. Although this approach may seem clumsy, the convenient mathematics of Fourier transforms makes it possible to establish an explicit relationship between $F(x,y)$ and the light intensity within the image, $I(x'y')$. The most important result of this treatment is that if the objective aperture could be infinite, the primary image of the object would be the square of the object transmission function, i.e.:

$$I(x',y') = C\,|F(x',y')|^2$$

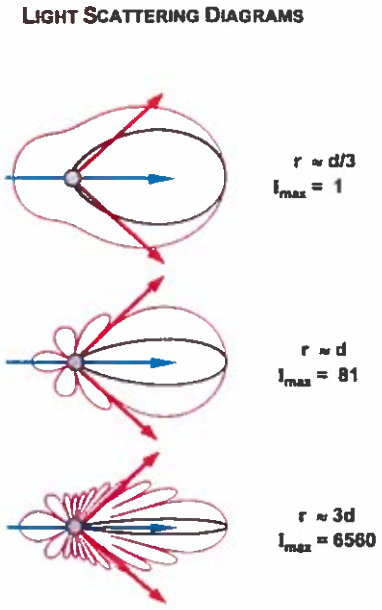
where the respective co-ordinates in the object and its image are related through the magnification between the object and its image: i.e. $x' = xM$, $y' = yM$ (Born & Wolf, 1980). Unfortunately, the wave pattern in the objective back focal plane is always partly cut off. In these circumstances the resultant image cannot be interpreted as $|F(x',y')|^2$ because the wave pattern observed in the image plane is merely the inverse Fourier transform of a wavefront that has been modulated by the objective aperture.

It may be useful to point out an intrinsic correspondence between Abbe's original theory and the Fourier optics approach. Any undulating component of the Fourier transform of the object transmission function $F(x,y)$ can be regarded as an individual diffraction grating contributing its particular diffracted wave to the overall wave pattern within the objective back focal plane (Saleh & Teich, 1991). If certain extremely deflected diffracted waves miss the acceptance cone of the objective, their corresponding 'diffraction gratings' cannot be seen in the image produced by the microscope. This means that all the object details of a size comparable to about half the spatial period of the eliminated 'grating' will disappear from the final image. Using the complete mathematical formalism of the Fourier theory the objective resolving power was estimated to be about $0.5\lambda/NA$ (Young, 1996).

Note that beyond the theory of image formation by light microscopes, Fourier transforms can also be found in digital image processing. In this case the Fourier transform of the digitised image is computed first, then modified by a proper mathematical filter, and finally the inverse Fourier transform is computed to re-form the object image. This method proved useful for improving and/or manipulating microscopical images (Inoué, 1986).

Fourier theory and image appearance

The general conclusions of the Fourier theory are obviously too abstract. However, the most important features of the modulation effect of the objective back focal plane including their role in the process of image formation can be grasped without computing the Fourier transforms of particular objects? What is the common key feature of Fourier images of miscellaneous microscopical objects? A clue is provided by the linear nature of Fourier transforms. This property means that for any object that can be considered to be composed of several well-defined object details an overall Fourier image is the sum of the Fourier transforms partial to these structural elements. We will consider as a structural detail any object feature that can exhibit detectable contrast of luminous intensity relative to its surroundings and thus can be visualised by the microscope. In general, the optical effects that are related to the propagation of light through material depend considerably on the size of investigated objects. From this point of view microscopical objects and/or their structural details can be broadly classified as small particle-like inhomogeneities and large smooth surfaces, either planar or curved ('small' denotes having a size about equal to the wavelength of light or less). The result of the interaction of light with small particles, such as fibres, microspheres and granules and/or various particle-like blebs and spicules sitting on larger surfaces, is usually referred to as light scattering (van de Hulst, 1957). When light impinges on large smooth surfaces, either plane or curved, the application of the Huygens-Fresnel principle leads to optical effects known as reflection and refraction (e.g. Benson, 1991). Next we will examine the properties of Fourier images of such simple structural details and try to link them to the performance of light microscopes. The light-scattering properties of small particles are usually presented as polar diagrams *(scattering diagrams)*, showing the dependence of diffracted light on the angle at which it has been deflected from the its original direction of propagation (Fig. 4).



Regardless of the particle shape and size its scattering diagram exhibits a central lobe in the direction of the original light beam, with some side lobes around it. If the particle size modestly exceeds the wavelength of light the main lobe is very narrow and intense. Then a substantial part of diffracted light and sufficient number of side lobes can match the acceptance angle of the objective even if its numerical aperture is relatively low. On decreasing the size of the particle, the forward diffraction lobe will broaden, gradually approaching a situation when its width considerably exceeds the acceptance angle of the objective. Thus it will resemble an undiffracted wave, such as the one coming from the specimen background. A particle that has produced such a wide-angle diffraction pattern cannot therefore be seen in the microscope with realistic shape and size. In a similar way to Abbe's conclusion concerning the images of diffraction gratings, the image of a small particle will resemble the original object according to the degree to which the side lobes of scattered light can enter the objective. For microspheres of radius equal to 0.61)/NA it is exactly the central scattering lobe that matches the acceptance angle of the objective.

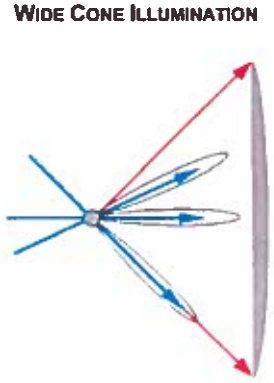
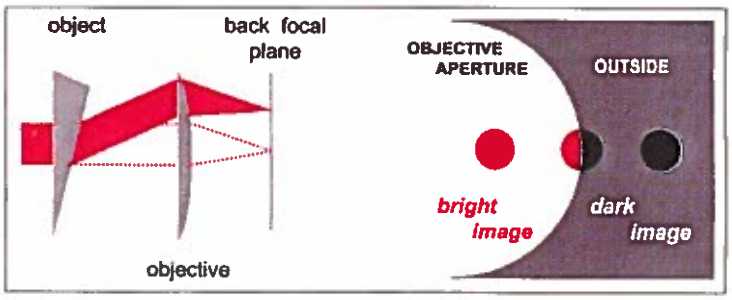


Fig.4. Light scattering by small spherical particles. Scattering diagrams were calculated within the framework of the Rayleigh-Gans approximation (van de Hulst, 1957) for transparent particles of three different radii that are related to the Rayleigh resolution limit, δ, of a NA = 0.7 objective as indicated in the figure; I_{max} , is the maximum intensity in the forward scattering lobe, presented in relative units (the absolute intensity of the scattered light will vary with the index of refraction of the particle as $I_{max} \sim [n-1]^2$). For opaque particles, Fraunhofer diffraction theory should be used instead of the Rayleigh-Gans approximation, but the result will not differ significantly from the data shown in the figure. The bottom diagram (wide cone illumination) illustrates why even transparent particles may appear dark in bright-field microscopy: part of the scattered light from the pencils of light close to the maximum acceptance angle of the objective is missing in the particle image, thus making it darker than its background. Blue arrows, incident light; black lines, linear-scale scattering diagrams; dark magenta, logarithmic scale scattering diagrams; red arrows, acceptance cone of objective.



The interpretation of the effects of light reflection and refraction by large structural details is even easier. The beams of illuminating light that have been deflected from their original direction by either of these two mechanisms will be focused to distinct points in the objective back focal plane (Fig. 1).

Depending on its particular tilt angle, the focused beam will either fall into the objective aperture or miss it (Fig. 5).

When the reflected or refracted beams fall within the acceptance angle of the objective, the surfaces at which they originated will be seen as very bright. In the opposite case the surface image may appear as much darker than the specimen background. This 'spatial filtration' of reflected and refracted light that takes place in the objective back focal plane is related to image contrast rather than to the resolving power of the microscope. Large surfaces whose characteristic dimensions exceed multiples of the wavelength are imaged faithfully because even an objective of low aperture could accept nearly all the 'diffracted' rays, provided that they have been reflected or refracted to the objective acceptance angle. When they miss the objective, the surface of concern is still visible, but appears dark.

14. Wide-cone illumination and oblique illumination

Illumination by very narrow cones of light is usually not generally employed in microscopy. Instead, condensers of high numerical aperture are used, illuminating the specimens with wide cones of light. Ernst Abbe treated wide-cone illumination in terms of a multitude of very narrow pencils of rays (Evennett, 1996). Following this conception, the wavefront in the objective back focal plane is a superposition of many Fourier images, each of them being associated with a particular pencil of light. As far as the quality of the ensuing object image is concerned, the most important difference between the constituent Fourier images stems from the obliquity of their corresponding illuminating light. Increasing the inclination angle of an arbitrary illuminating pencil, a focus formed by the un-diffracted light will move from the central position (typical of axial illumination) towards the periphery of the objective aperture (Fig. 1). Under such a condition the objective may also accept highly diffracted light that would be cast outside the objective aperture if axial (narrow-cone) illumination is used. Object features can thus be seen in the image, which are smaller than with pure axial illumination, an interesting finding which dates back to Abbe's original work on diffraction (Abbe, 1873). Oblique, or *anaxial,* illumination can be treated as an asymmetric expansion of the acceptance cone of the objective which leads to an improved resolving power, or *super-resolution* (Rhodes, 1953; Kachar, 1985; Greenberg & Boyde, 1997). In certain microscopes the condenser is constructed to ensure that only oblique illumination is used, e.g. *dark-field, phase contrast and Hoffman modulation contrast microscopy.* In these cases the primary goal of oblique illumination is to generate enough image contrast for visualising non-absorbing phase objects, while super-resolution is noted as a spin-off effect only. An instrument in which oblique illumination has been carefully employed to improve the practical resolution is the direct-view 3-D microscope of Greenberg & Boyde (1997).

Acknowledgements

This work was supported by grants from the Czech Grant Agency (202/97/112 1) and TEMPUS (IMG-96CZ-2019). We are also indebted to S. Damjanovich and G. Dunn for their encouragement, which stimulated greatly the preparation of this article, and to P. Evennett for very helpful editorial work.

References

Abbe, E. (1873) Beitr5ge zur Theorie des Mikroskops und der mikroskopischen Wahrnehmung.*Arch.f mikroskop.Anatom.* 9, 414-468.

Benson, H.(1991)*University Physics.*Wiley,NewYork.

Born, M. & Wolf, E. (1980) *Principles of Optics, 6th edn.* Pergamon Press, London.

Castleman, K.R. (1979) *Digital Image Processing.* Prentice-Hall Inc., New Jersey.

Cherry, R.J. (ed.) (1991*) New Techniques of Optical Microscopy and Micro-spectroscopy.* Macmillan, London.

Evennett, P. (1996) The First Ernst Abbe Lecture: Abbe and the development of the modern microscope. *Proc. Royal Microsc. Soc.,* 31(4), 283-292.

Françon, M. (1961) *Progress in Microscopy.* Rowe Peterson & Co., Evanston.

Goodman, J.W. (1988) *Introduction to Fourier Optics. 2nd edn.* McGraw-Hill, New York

Greenberg, G. L. & Boyde, A. (1997) Convenient and controllable direct-view 3D imaging in conventional light microscopes: approaches via illumination and inspection. *Proc. Royal Microsc. Soc.,* 32(2):87-101.

Hopkins, H.H. & Barham, P.M. (I 950) The influence of the condenser on microscopic resolution. *Proc. Physical Soc. B.* 63, 737-744.

Inoue, S. (1986) *Video Microscopy.* Plenum Press, New York.

Kachar, B. (1985) Asymmetric illumination contrast: a new method of image formation for video light microscopy. *Science* 227, 766-768.

Kapitza, H.G. (1996) Modern microscope optics. *Proc. Royal Microsc. Soc.* 31(4), 300-303.

K6hler, A. (1904) Mikrophotographische Untersuchungen mit ultraviolettem Licht. *Z. Wiss. Mikrosk.* 21, 129-165.

Lacey, A. J. (1989) The principles and aims of light microscopy. In: Lacey, A.J. (ed.). *Light Microscopy in Biology. A Practical Approach.* IRL Press, Oxford.

Martin, L.C. (1966) *The Theory of the Microscope.* Blackie, London.

Pauluzzi, I., Lichtscheidl, I.K. & Url, W.G. (1996) Ultra-violet microscopy. *Proc. Royal Microsc. Soc.* 31(2), 155-160.

Pawley, J. B. (ed.) (1995) *Handbook of Confocal Microscopy 2nd edn.* Plenum Press, New York.

Pluta, M. (1988) *Advances in Light Microscopy. Principles and Basic Properties. Vol.* 1, Elsevier, Amsterdam.

Rhodes, J.E. (1953) Microscope imagery as carrier communication. *Jour. Opt. Soc. Am.* 43, 848-852.

Saleh, B.E.A. & Teich, M.C. (1991*) Fundamentals of Photonics.* J.Wiley, New York.

Shotton, D. (ed.) (1993) *Electronic Light Microscopy.* Wiley Liss, New York.

van de Hulst, H.C. (1957) *Light Scattering by Small Particles.* J.Wiley, New York.

Young, 1. T. (1989) Image fidelity: characterizing the imaging transfer function. *Methods Cell Biol.* 30B, 1-45.

Young, 1. T. (1996) Quantitative microscopy. *IEEE Engineering in Medicine and Biology 15(i),* 59-66.

Zernike, F. (1955) How I discovered phase contrast. *Science,* 121,345-349.