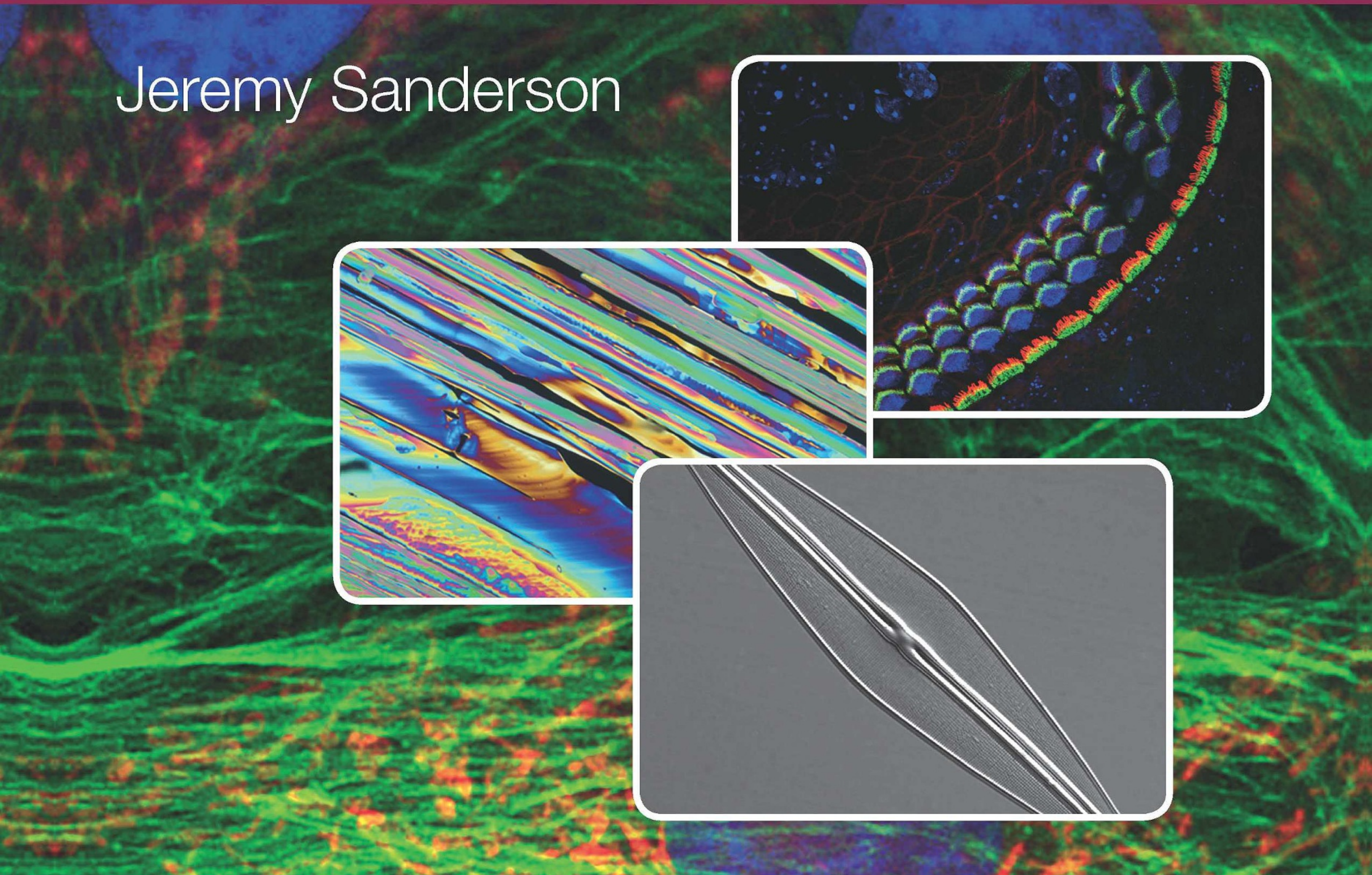
UNDERSTANDING LIGHT MICROSCOPY

Jeremy Sanderson



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Understanding Light Microscopy

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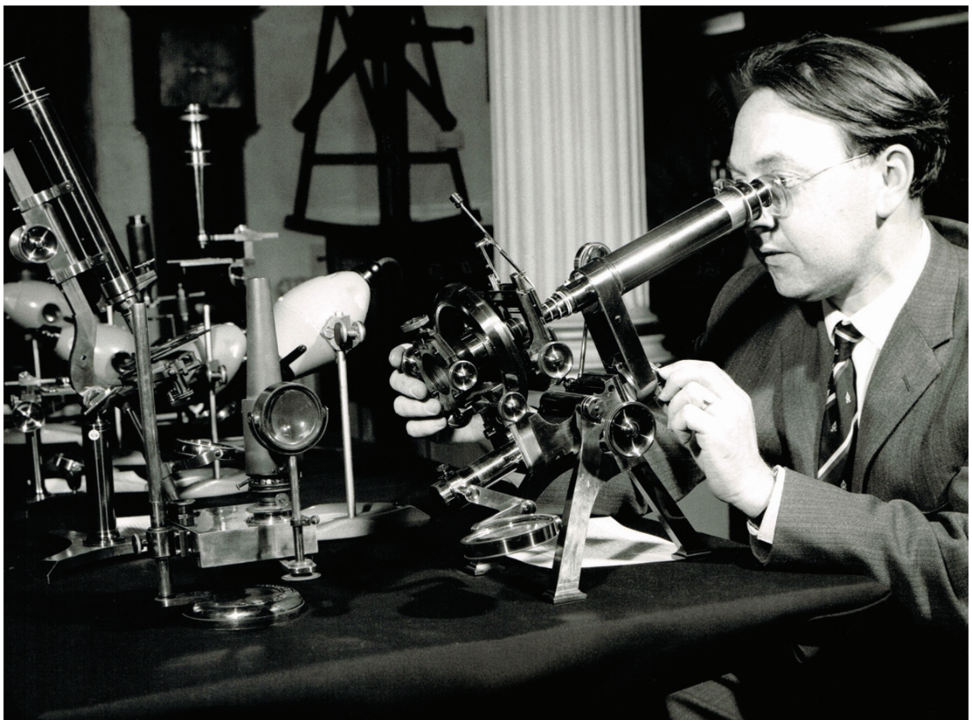
Dedicated to the memory of:

Savile Bradbury
6th February 1931 – 29th November 2001

Teacher, mentor, friend



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About the Author

Jeremy Sanderson is the Bio-imaging Facility Manager at MRC Harwell Institute, and a Chartered Scientist. He has been working in core imaging facilities for 16 years, and as a light and electron microscopist for over three decades. He joined the Royal Microscopical Society in 1987, has taught regularly on its instruction courses; has also served on the RMS Council, and assists with RMS teaching qualifications and educational outreach. Starting as an NHS histologist, Mr Sanderson then worked at Oxford University before going on to the Max Planck Institute of Molecular Cell Biology and Genetics in Dresden. From there he returned to Sheffield University to set up and manage a light microscopy imaging facility before joining the Medical Research Council in 2009.

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The world of the professional and amateur microscopist is a select one; more importantly it is filled with friendly and helpful folk whom I count fortunate to have as friends and colleagues. The microscope is a wonderful instrument, which I hope you will realise from the pages of this book. To the very competent and highly professional staff in the Royal Microscopical Society office, who have helped me considerably over the years, as well as friends and colleagues in the wider microscopical *Gemeinschaft*: thank you all. A good friend, thank you Clive, has suggested – tongue in cheek – that I ought also to explain in this book what a microscope *cannot* do. Whilst the light microscope is an extremely sophisticated and versatile instrument capable of many things, it cannot unfortunately solve political or relationship issues, nor the deep spiritual or philosophical questions of humankind. That said, it will reveal to you a wondrous world of superb order of the infinitesimally small.

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Of course, any errors of commission or omission are mine alone, not the responsibility of the above-named. I welcome comments and criticism for improvement of this book, and possibly encouragement (which we all thrive on) should you wish to give it.

Look-Up Guide to Feature Boxes by Theme

Autobiographical Boxes

Information Boxes

Exercise Boxes

Glossary and Definitions

Aberration
An optical defect inherent in the design of a lens causing an optical system not to form a perfect image.

Absorption
The transformation of radiant energy (e.g. light) to a different form of energy (typically heat) by interaction with matter. This gives rise to colour when absorption is wavelength dependent. At the atomic level, quanta of energy are transferred from the radiant energy field (e.g. light beam) to electrons within atoms and molecules, causing attenuation of the radiant energy.

Acceptance angle
The angle between the extreme rays of light collected by an objective lens (see angular aperture).

Accommodation
The act of adjusting the refractive power of the eye to allow sharp imaging of an object located at a particular distance.

Achromatic
Free of colour. Absence of chromatic aberration.

Achromatic lens
A lens, or microscope objective, comprising two or more elements (usually of crown and flint glass) corrected for chromatic aberration, i.e. to have the same focal length with respect to two or more selected colours (e.g. blue and red). Such a lens is also corrected for spherical aberration and other aperture-dependent defects with respect to one (e.g. green) or more colours (see also apochromatic lens and semi-apochromatic lens).

Acousto-optical tunable filter (AOTF)
Used in microscopy as a tunable bandpass filter. An AOTF comprises a tellurium dioxide or quartz anisotropic crystal to which a piezoelectric transducer is bonded. Radio signals from 40–70 MHz produce a controllable acoustic wave through the crystal in the visible spectrum causing it to act as a diffraction grating with a bandpass characteristic.

Aerial image
A real image existing in a plane in space, not normally visible to the eye or a camera or other detector.

Airy disc and Airy pattern
Diffraction of a point source of light at a circular lens aperture forms a central bright disc surrounded by a sequence of concentric alternating dark and light rings, the entirety of which is called an Airy pattern. The diameter of the pattern depends upon the wavelength of the illuminating light and the diameter of the circular aperture. The central part of this pattern is called the Airy disc. It is the smallest point to which a beam of light can be focused. A point source will always form an Airy pattern.

Amplitude
The strength of a wave field at a particular location and instant, referring here to the electric field of light.

Angular aperture
Half the maximum plane angle subtended by a lens at the centre of either an object field or image field by two opposite marginal rays when the lens is used in its correct working position.

NB. The *RMS Dictionary of Light Microscopy* notes: sometimes the full plane angle is taken as the angular aperture, but this causes certain inconveniences in geometrical optical or technical lighting calculations. The acceptance angle (see above) defines the full plane angle subtended by a lens.

Aperture
A fixed or variable opening through which light may pass. Aperture may also, in context, refer as a shortened name for numerical aperture. See also Note 2 in the Notes section.

Aperture diaphragm
A diaphragm in the plane of an aperture of an optical system which delimits its pupil, or cross section of all rays bundles of light. This term usually refers specifically to the iris diaphragm situated in the illumination set of conjugate planes of a microscope and is usually located beneath the microscope condenser in its front focal plane, where it controls the angle of convergence of the solid cone of light emerging from the condenser.

Aplanatic
Corrected for spherical aberration and coma by complying with the sine condition (see sine condition).

Apochromatic lens
A lens, or microscope objective, in which axial chromatic aberration is minimised for at least three wavelengths, the sine condition fulfilled for two wavelengths and spherical aberration and other aperture-dependent defects minimised with respect to one (e.g. green) or more colours. The most highly corrected of the three classes of objective (see also achromatic lens and semi-apochromatic lens).

Back focal plane
The second, or rear, focal plane of a lens. It usually refers specifically to the objective where all rays diffracted by interaction of light with the object pass. The back focal plane of the objective is one of the illumination, or aperture, set of conjugate focal planes, which also include the lamp filament; the aperture diaphragm in the front focal plane of the condenser and the exit pupil of the eyepiece.

Bandwidth
The difference between the upper and lower limits of a frequency band. The amount of data transmitted at a fixed rate, usually expressed in hertz. May also be used to cite the part of the spectrum passed by a filter, in which case the units are nanometres.

Baud rate
Number of bits per second at which a digital signal is transmitted between computers.

Beam
A beam is a group of parallel, or approximately unidirectional, rays (see also ray).

Bertrand lens
A supplementary lens system that may be introduced into the microscope body-tube above the objective to transfer an image of the back focal plane of the objective into the primary image plane of the microscope. Together with the eyepiece the Bertrand lens forms a viewing telescope for inspecting the back focal plane of the objective. Sometimes called an Amici-Bertrand lens. Used for conoscopic observation in polarised light microscopy and adjustment of the illumination system, especially in conjunction with phase-contrast microscopy. Named after Émile Bertrand, the 19th century French mineralogist.

Bioluminescence
The production and emission of 'cold' light by a living organism, as a result of the enzymatic catalysis of the oxidation of luciferin. The enzyme is either luciferase or a photoprotein (e.g. GFP).

Birefringence
The property of possessing different refractive indexes with respect to light polarised in different azimuths set orthogonally to one another. For optically uniaxial media, the difference between the extraordinary principal refractive index and the ordinary one, viz: $\Delta n = n_e - n_o$. The quantitative expression of the maximum difference in refractive index due to double refraction.

Bit
A contraction of the words 'binary digit'. A bit is the smallest unit of data: it is either on or off, black or white, a 'one' or a 'zero'. Eight bits are arranged into one byte of information. A bitmap is a spatially mapped array of bits comprising a raster image (see also raster image).

Bit depth
The number of bits used to represent intensity values of an analogue stream of photons converted to a digitised value at each sampling point as a discrete pixel, or 'picture element' which forms a raster image.

Bright-field
Microscopy or illumination system in which the direct (i.e. zero order) light passes through the objective aperture and illuminates the background against which the image is seen. It is the most usual form of transmitted-light microscopy.

Brightness
The visual perception of a source which appears to be radiating or reflecting light, elicited by the luminance of a visual target. Brightness is not necessarily proportional to luminance. The electric vector of light is the parameter which causes the sensation of brightness. Brightness should not be used to describe illuminance or radiance (see also light intensity and Chapter 1, endnote 1).

Cardinal distances
In geometrical optics the distances between the cardinal points of a lens. These are: the focal lengths *f* on the object and *f*′ on the image side; the object distance, u, and image distance, v; the intersection distances (s and s′) between the vertex and the object/image points respectively; the hiatus between the principal points. The vertex is the intersection of the surface of the lens with the optical axis. The hiatus is the distance along the optical axis between the principal planes h and h′ of a lens.

Cardinal points
The reference points on the axis of a lens. These are: the focal points, F and F′; the principal points, H and H′; the nodal points, N and N′; the object point, O, and the image point, I or O′.

Chromatic aberration
An inherent optical defect of a lens, due to dispersion by the material from which it is made, which causes different colours to be brought to a focus at different distances from the lens. It degrades image quality and may be seen as colour fringes surrounding edges of features in the image. This aberration is corrected, or minimised, by combining lens elements from glass, or other materials, of different dispersive power.

Coherence
The correspondence, or equivalence, of different wave trains of light of relative phase emanating from a common source, which permit the waves to interfere. Coherent radiation can be produced only by a very small (point) source, by a very small part of a larger source – effectively a point source – or by a laser (see also incoherence).

Collimation
The process of aligning two or more optical axes with respect to one another, or to a reference axis.
The act of making rays originating from one point parallel to one another. This is usually done with a converging lens or a concave mirror.

Compensator
A retardation plate of variable optical path difference, itself used to measure optical path-length differences within a birefringent (i.e. doubly-refracting) specimen.

Condenser
A single converging (positive) lens, or a group of such lenses, used in the illuminating path to collect light and concentrate it by convergence into the specimen plane and evenly illuminate the object being examined.

Condenser diaphragm
The iris diaphragm usually located beneath the microscope condenser in its front focal plane, situated in the illumination set of conjugate planes of a microscope, where it controls the angle of convergence of the solid cone of light emerging from the condenser. Also sometimes referred to as the illuminating aperture iris (IAI) or illuminating aperture diaphragm (IAD).

Conjugate plane(s)
Planes in an optical system that are equivalent such that an object placed in one plane will be imaged sharply at each subsequent plane in that series. Two separate sets of conjugate planes – the Aperture (illumination) set and the Field (imaging) set exist in the microscope. These planes are perpendicular to the optical axis at the conjugate points of an optical system; the conjugate points being those points in both object space and image space which are imaged upon one another.

Conoscopic image
The interference pattern and diffraction image seen at the back aperture of the objective in the rear focal plane.

Contrast
The difference in tonal intensity, brightness or colour from point to point within the object or image, and variations in the intensity ratio between the object and its background as transferred into the image.

Contrast transfer function (CTF)
As regards the modulation transfer function (MTF) when the brightness of the periodic object varies as square wave, the MTF becomes the CTF (see also MTF). A graphical measurement of the ability of an imaging system to transfer contrast from the object to the image.

Convolution
The mathematical operation on two functions to produce a third resultant function. In microscopy the resulting image of a complex object is the linear shift-invariant convolution of the true object and the point spread function through a dioptric lens (see also deconvolution and point spread function, PSF).

Correction collar
A graduated scale fitted to the barrel of a high numerical aperture dry objective, or sometimes to a long working distance or immersion objective, that acts to move internal lens elements to correct or minimise spherical aberration introduced by the thickness of the coverslip and/or refractive index mismatch between the specimen preparation and immersion medium.

Cut-off frequency
Diffraction at the image plane causes all imaging systems to act as low pass filters with a finite ability to resolve detail. This is the cut-off frequency, k_{max}, which is equal to $2NA/\lambda$ shown on an OTF plot as the Abbe limit: the smallest spatial frequency in the object that can be transferred into, and discriminated in, the image. For fluorescence microscopy, λ_{em} is used (see also optical transfer function).

Dark-field
Microscopy or illumination system in which the direct light is prevented from passing through the objective aperture. The image is formed from light scattered and diffracted by features in the object, the detail appearing bright against a dark background (see also bright-field).

Deconvolution
Inverse of the convolution operation. In fluorescence microscopy, using a calculated or experimentally-derived point spread function to computationally recover an image degraded by the process of convolution.

Depth of field
The depth of sharpness in object space; the axial depth of the space on both sides of the object plane within which the object can be moved without detectable loss of sharpness in the image and within which features of the object appear acceptably sharp in the image while the position of the image plane is maintained.

Depth of focus
The depth of sharpness in image space; the axial depth of the space on both sides of the image plane within which the image appears acceptably sharp while the positions of the object plane and the objective are maintained.

Dichromatic mirror
Also known as a dichroic mirror, the essential component of an epi-fluorescence microscope, an interference filter which selectively reflects shorter-wavelength excitation radiation whilst simultaneously transmitting the longer-wavelength emission fluorescent signal.

Diffraction
The spreading of light after passing the edge of an object, or after passing through a small aperture, particularly features that act as such within the object from which an image is formed.

Dioptre
Unit of refractive power expressed as the reciprocal of the focal length of a lens in metres. See also Note 4 in the Notes section.

Direct light
Light that enters the objective which undergoes no change in propagation direction after passing through the object field or being specularly reflected off the surface of the object. It gives rise to the zero-order of the diffraction image formed in the back focal plane of the objective.

Dispersion
The change in phase velocity of a wave train as a function of wavelength when passing from one medium to another, causing the separation of white light or several colours into individual component spectral colours. The phenomenon whereby the refractive index of optical glass varies with wavelength.

Dynamic range
The number of values in a signal that may be individually distinguished, expressed as a ratio or logarithmic value (e.g. decibels) of the difference between the smallest and largest signal values.

Emission
Release of radiant energy. Conversion of the energy of an excited atom or molecule into a photon.

Empty magnification
High magnification that increases the size of an image without any concomitant increase in resolution, due to a limitation upon the resolving power of the optical system.

Entrance pupil
The apparent minimum cross section of all ray bundles on the object side of a lens.

Excitation
The input (sometimes by absorption) of energy into matter leading to the emission of radiation.

Exit pupil
Point at which bundles of refracted rays emerging from a lens on the image side cross over (i.e. at its back focal plane). In the case of the microscope eyepiece, the point where the observer's eye should be located.

Extinction
The condition whereby an optically anisotropic specimen appears dark when viewed between crossed polars. The extinction factor is a measure of the perfection of extinction with crossed polars, expressed as a ratio of intensities: $X_{\parallel}/X_{\perp}$.

Eye relief
The distance between the eye lens of the microscope eyepiece to the eye point, or exit pupil of the microscope.

Field diaphragm
A diaphragm limiting the field of view, usually fitted just after the lamp collector lens or at the primary image plane in the eyepiece. The term normally refers to the illuminated field diaphragm (IFD) situated in the base of a modern microscope, which is a member of the field, or imaging, set of conjugate planes, which also include the specimen plane; the primary image plane and the retina or camera detector.

Field of view
The visible area seen through the microscope when the specimen is in focus. The greater the magnification, the smaller the field of view, and vice versa.

Fluorescence
The phenomenon of selective absorption of quanta of short-wavelength radiation and re-emission of signal at a longer wavelength. A form of photoluminescence.

Fluorite objective
A semi-apochromatic lens objective intermediate in its correction between an achromat and apochromat. So-called because fluorite, having low dispersion, was once used in the construction of these and apochromatic objectives to minimise and thus correct for chromatic aberration. Fluorite objectives are often colloquially referred to by brand names, such as 'Neofluar' or 'Fluotar' (see also semi-apochromatic lens).

Focal length
The distance, f or f', measured along the optical axis from the principal plane of the lens, objective, or group of lenses, to the focal point.

Focal plane
The plane at right angles to the optical axis at the focal point.

Focal point
The point where light rays originating from a distant source set at infinity come to a focus and form an image after passing through a lens. If the source is set at the focal point, these rays emerge parallel to each other after passing through the lens.

Gain
The numerical factor by which an amplifier increases signal strength. In a light detector the number of electrons counted per detected photon. In a CCD or sCMOS camera, this factor is unity. In a photomultiplier tube (PMT) the gain ranges from 1 to $\approx 10^6$.

Gamma
The function relating digital intensity to display brightness. A nonlinear operation used to encode and decode luminance values since these are not generally a linear function of the input signal.

Glare
Non image-forming stray light, which diminishes contrast. Such scattered stray light generally arises from the improper adjustment or use of field or aperture diaphragms (see also stray light).

Graticule
A pattern such as a scale or a grid, together with its support, which is placed in an object or image plane within the microscope or optical system. It is used for measurement, alignment, location reference or stereological analysis. Most commonly situated at the primary image plane in an eyepiece. Also sometimes referred to as a reticle.

Illuminated field diaphragm (IFD)
An adjustable iris diaphragm limiting the field of view situated in the base of a modern microscope, which is a member of the field, or imaging, set of conjugate planes, which also include the specimen plane; the primary image plane and the retina or camera detector.

Illuminating aperture diaphragm (IAD)
An adjustable iris diaphragm situated in the illumination set of conjugate planes of a microscope located in the front focal plane of the condenser, where it controls the angle of convergence of the solid cone of light emerging from the condenser. Sometimes called the IAI, or illuminating aperture iris.

Image
A representation, necessarily less than perfect, of an object made by a lens or mirror, which is located in another plane, formed by means of light (photons) or other form of radiation (e.g. electrons).

Incoherence
An adjective expressing the property of light waves not satisfying the condition of coherence and thus being incapable of interference (see also coherence). The lack of a fixed phase relationship between two, or more, waves.

Interference
Alteration of the total intensity of light and the amplitude sum produced by the interaction of two coherent light waves, either constructively or destructively. The phenomenon is used to convert optical path-length differences in the object into intensity variations in the image, thus providing contrast.

Köhler illumination
A system devised by August Köhler for producing even illumination of the specimen using small uneven light sources, often possessing a definite filament structure, which would otherwise interfere with observation and/or analysis of the image.

Light Intensity
Luminous intensity is the perceived power per unit solid angle, measured in candelas (cd = lumens/steradian), whereas is luminous flux is the total perceived power emitted in *all* directions; it is measured in lumens and is proportional to the square of the amplitude of the light wave. Illuminance is the luminous flux incident upon a surface and luminous excitance the luminous flux emitted from a surface, both being measured in lux (lumens/m^2). Luminance, measured in cd/m^2, is luminous flux per unit solid angle per unit projected source area. Light output is expressed in lumens, whereas light intensity is measured in lux (see also brightness and Chapter 1, endnote 1).

Magnifying power
The ability of an optical system to produce a magnified image, usually expressed as the lateral or angular magnification of the image (see also Note 1 in the Notes section). When applied to the objective, the term refers to the lateral magnification of the primary real image by the objective alone when corrected for a finite image distance, or by an infinity-corrected objective and its associated tube-lens together. When quoted for an infinity-corrected objective without the tube-lens, the angular magnification is given (e.g. 40x).

Mechanical tubelength
The distance between the shoulder of the objective where it screws into the nosepiece and the top of the microscope body-tube into which the eyepiece fits: the length of the body-tube in its simplest form without any intermediate lenses. This distance is commonly 160 mm for finite tubelength objectives, whereas for infinity-corrected objectives the tubelength is hypothetically considered to be infinite.

Modulation transfer function (MTF)
Mathematical function describing the ability of an optical or electronic device to transfer a signal as a function of the spatial or temporal frequency of that signal (see also CTF, OTF).

Monochromator
Any device used for isolating portions of the spectral output of a complex radiant energy (e.g. light) source.

Noise
Random fluctuations in the signal intensity recorded in an image that are not due to the object being imaged. Unwanted interference in a signal or display. Particularly prevalent in low-light conditions; shot noise is random noise due to quantum uncertainties when small numbers of photons are recorded (see also signal-to-noise ratio).

Numerical aperture (NA)
A measure $n.\sin\alpha$ of the light-gathering power of the objective or condenser, devised by Ernst Abbe, where n is the refractive index of the medium between the front lens of the objective and the specimen preparation, and α is the half-angle (see acceptance angle) of the cone of rays entering the objective. This NA value is usually found engraved on objectives, and sometimes the condenser, and is a factor in the mathematical formula expressing the resolving power of the microscope. The greater the numerical aperture value, the greater the resolving power and also the light flux gathered by the objective.

Objective
The principal part of the microscope, consisting of a lens, or a series of lens elements, its mount and any associated parts. The objective (together with a separate tube-lens in an infinity-corrected microscope) forms the primary image. The NA of the objective is a fundamental parameter (see also numerical aperture).

Optical axis
A straight line joining the centres of curvature of lens surfaces.

Optical index
A term devised by Edward Nelson, being the ratio of the numerical aperture to the magnifying power of an objective. The OI describes mathematically how closely information is packed into the primary image. It can be used to ensure that the analogue image is sampled adequately by a digital camera. As the optical index increases, the sampling requirements become more stringent.

Optical indicatrix
The visual representation of refractive index by an imaginary spatial figure in the shape of an ellipsoid of revolution, or triaxial ellipsoid, representing the refractive indexes of a doubly refracting material as distances between the centre of the ellipsoid and points on its surface and the vibration of plane-polarised light related to the refractive indexes by the directions of the said distances. Not to be confused with a ray velocity surface representation.

Optical transfer function (OTF)
The optical transfer function plot illustrates graphically how any imaging system transfers resolvable object information into the image. It is a combination of the modular transfer function (MTF) and the phase transfer function (PTF). The MTF describes how the imaging system handles both resolution and contrast; the PTF describes the inherent aberrations. Conventionally, the OTF is normalised to unity for zero spatial frequency. Strictly, the Fourier transform of the PSF is equivalent to the OTF, providing the phase component of the optical transfer function is negligible (see also CTF, cut-off frequency, MTF).

Optical tubelength
Distance between the back focal plane of the objective and the primary image plane (the front focal plane of the eyepiece). Relevant only to microscopes fitted with objectives corrected for a *finite* primary image distance.

Overcorrection
Error in the correction of spherical aberration leading to an unsharp image lacking in contrast. Caused by using a thicker coverslip or an optical tubelength longer than intended by the designer when computing the objective used. May be corrected using a correction collar, if fitted (see also undercorrection).

Parcentral
The quality applied to objectives grouped on the nosepiece of the microscope such that a particular specimen detail in the centre of the field of view with one objective remains centrally placed when another objective of different magnifying power is rotated into position on the optical axis.

Parfocal
The quality applied to objectives such that practically no change of focus has to be made when an objective of one magnifying power and depth of field is substituted for another.

Photon
Massless quantum particle of radiant energy of the electromagnetic field, whose value, E, is equal to hν.

Pixel
A portmanteau of the words 'picture element'. A small two dimensional geometrically-square non-overlapping Cartesian subunit, the smallest addressable sampling point forming a raster image. A voxel is the three dimensional equivalent.

Point spread function (PSF)
The impulse response of a focused imaging system to a point source or point object. The PSF is a spatial description of the optical transfer function of the imaging system: i.e. the signature of the microscope. The intensity distribution in the image from a convolved sub-diffraction point source. In incoherent imaging systems (e.g. fluorescence microscopes) the image formation process is linear.

Primary image
The enlarged real image of the specimen formed by the objective lens. The primary image plane (PIP) being the one of the imaging (field) set of conjugate planes, and where the primary image is formed.

Principal focus
The focal point for a beam of light rays parallel to the optical axis when refracted by a lens or reflected by a mirror.

Principal plane
For a thin lens, the plane perpendicular to the optical axis passing through the centre of the lens and from which the focal lengths are measured. A thick lens has two principal planes separated by the hiatus. The principal point(s) occur where the principal plane(s) intersect the optical axis.

Pupil
Cross section of all rays bundles of light.

Ramsden disc
Older term used for the exit pupil of the eyepiece.

Raster
The pattern of lines or points traced, or systematically applied to an area by rectilinear scanning, most commonly in microscopy by a laser beam regularly and repeatedly sampling the specimen.

Raster image
An image composed of pixels.

Ray
A geometrical abstraction; a straight line describing the direction of propagation of radiation, which in an isotropic medium is normal to the propagated wavefront. Ray bundles are all those rays bounded by a diaphragm. A beam is a group of parallel, or approximately unidirectional, rays.

Real image
An image which can be formed on a screen.

Rear focal plane
The second, or back, focal plane of a lens. It usually refers specifically to the objective where all rays diffracted by interaction of light with the object pass. The rear focal plane of the objective is one of the illumination, or aperture, set of conjugate focal planes, which also include the lamp filament; the aperture diaphragm in the front focal plane of the condenser and the exit pupil of the eyepiece.

Refraction
The change in velocity of light (with or without a change in direction) upon crossing the boundary, or interface, between media of different refractive indexes. A change in the direction of propagation of radiation determined by the change in velocity of propagation upon passing through an optically non-homogeneous medium or upon passing from one medium to another in a direction other than normal to the interface.

Refractive index
The ratio (n) of the speed of light in a vacuum (c) to that given in a given medium n = c/v.

Resolution
The smallest distance between two points in an object that can just be distinguished as separate points in the image.

Resolving power
The ability of a microscope, or of an individual objective, to resolve or make visible fine detail present within the object in the image.

Reticle
A pattern such as a scale or a grid, together with its support, that is placed in an object or image plane within the microscope or optical system (see also graticule).

Sampling
The process of transforming a continuous analogue signal into a finite set of discrete digital values; each sampling point commonly being represented by an individual pixel or voxel. An 8-bit pixel, the most common type, allows for 256 (2^8) intensity tones of black, grey and white values.

Semi-apochromatic lens
A lens, or microscope objective, intermediate in its correction and complexity of construction between an achromat and apochromat (see also fluorite objective, achromatic lens and apochromatic lens).

Signal-to-background ratio (SBR)
A measure of contrast in the image. The ratio of image signal versus the average background level. Optical sectioning removes the background contribution to the image, giving higher SBR values.

Signal-to-noise ratio (SNR)
The ratio of intensity of the object represented in the image as signal versus the average noise level, which consists of unwanted, random, variation in intensity (i.e. noise) per sampling point not directly generated by the object.

Sine condition
A mathematical formulation derived by Ernst Abbe (and sometimes called the Abbe sine condition) of a condition that must be fulfilled by an aplanatic lens or other optical system to produce sharp images together of objects that lie off-axis as well as those that lie on-axis. Such an aplanatic system is free from both spherical aberration and coma.

Spectrum
The orderly arrangement of the dispersion of radiant energy derived from atomic or molecular vibration arranged by wavelength.

Spherical aberration
An inherent optical failure of a lens to form a sharp image due to the curvature, or 'figure' of the lens surfaces, which refract the rays of light such that peripheral and axial rays are brought to a focus at different distances from the lens. Commonly the peripheral rays are brought to a focus closer to the lens than the axial rays. Like chromatic aberration, spherical aberration degrades image quality; these two aberrations being the most serious the lens designer has to contend with.

Stop
A term used loosely for a diaphragm of fixed size (see also Note 2 in the Notes section).

Stray light
Light that does not contribute to image formation but arises from the scattering or reflection of light by the object, in lenses, or by obstacles in the light path and which reduces contrast in the image (see also glare).

Tubelength
See mechanical tubelength and optical tubelength.

Tube-lens
An intermediate lens designed as the last element of an infinity-corrected objective, located in either the body of the microscope tube or the viewing tube. The tube-lens is remote from the objective to allow any extra equipment conveniently to be placed in the parallel light path whilst maintaining the primary image at its proper location.

Undercorrection
Error in the correction of spherical aberration leading to an unsharp image lacking in contrast. Caused by using a thinner coverslip or mechanical tubelength shorter than intended by the designer when computing the objective used. May be corrected using a correction collar, if fitted (see also overcorrection).

Virtual image
An image that cannot be formed on a surface but which may be converted to a real image by the eye, or other converging lens system.

Visual acuity
Refers to the sharpness and clarity of vision and our ability to resolve, or distinguish, fine detail with the naked eye.

Voxel
A 3D sampling element (see also pixel).

Wave train
A sequence of light waves of equal phase, amplitude, wavelength and polarisation state travelling in the same direction with equal velocity.

Widefield
Microscopy whereby the entire field of view is imaged at once, as opposed to confocal fluorescence microscopy where the image is acquired by raster scanning of point illumination. The term is almost always used when referring to fluorescence microscopy.

Working distance
The free distance between the objective front lens and the upper surface of the coverslip, or the specimen if uncovered, when the latter is in focus. Generally, the greater the magnifying power of the objective, the smaller the working distance.

These definitions have been compiled from several sources, but many have been taken verbatim from the RMS Dictionary of Light Microscopy (1989) RMS Handbook No. 15, compiled by the Nomenclature Committee of the RMS, namely: Drs Bradbury, Evennett, Haselmann & Piller.

Notes

1. Magnification
The magnifying powers of objectives are cited in this book as 40x throughout for consistency. This is the correct notation for infinity-corrected objectives and also accessory optical components, such as the magnifying glass, magnification changers and eyepieces. In these cases, magnification is an angular measure. With a magnifying glass or eyepiece, our eyes act as the 'tube-lens' to form a real image from a lens which would otherwise form a virtual image. Because an infinity-corrected lens does not form a real image, it must be compared with something, and the focal length of the tube-lens (e.g. 180 mm) is chosen as the reference distance with which to compare its focal length. 40x thus describes the magnification of a 4.5 mm focal length objective (i.e. 180/4.5). However, for the older finite tubelength microscopes, with agreed standard tubelengths of 160 mm (sometimes 170 mm) and 250 mm, for biological and metallurgical stands respectively, magnification is a ratio of the image distance from the centre of the lens to the object distance from the same lens centre, and the proper notation should be 40:1. See Chapter 3 for more about geometrical optics.

2. Aperture
Apertures are associated with angles; they are not merely holes but the area of a lens that passes light. Therefore, for a builder to refer to an aperture in a wall is wrong: it is a hole. Informally 'aperture' is used to describe numerical aperture (usually of the objective), when the meaning is clear. A diaphragm may (i.e. in front focal plane of the condenser), or may not (i.e. at the illuminated field diaphragm) be an aperture diaphragm. A stop usually refers (loosely) to a diaphragm of fixed aperture; strictly it is an opaque area concentric with the optical axis. As such, a stop is used in dark-field illumination.

3. Spelling
This book is written by an Englishman who eschews Windows spell-checkers. The letter s is therefore used in place of z, as appropriate. Shakespeare detested z, describing it as unnecessary (*King Lear*, Act 2, scene 2, line 65). Many of my friends are American, and so I ask the forbearance and understanding of my American readers. A mere slip of Coleridge's pen must have spelt artefact when he coined the term in 1834. The stem of the third declension noun *ars* in compound words is *arti*-. Some books put a diacritic acute *accent aigu* on Abbe's name, thus: Abbé. He may have been a key figure in – arguably – the finest optical company ever and consequently a great High Priest of imaging, but Abbe was never a French abbot. Authors are fallible and make mistakes, but this one is fundamental. Any microscopy book that commits this sin should be shunned.

4. Terminology
The Royal Microscopical Society's *Dictionary of Light Microscopy*, although over 25 years old at the time of publication, is a very useful and little-known resource, which has been followed herein. Language both alters and evolves; words and meanings change subtly over time. For example, in the dictionary the term 'darkground' is favoured over dark-field (as opposed to bright-field). Some hold that the term should properly be darkground, since if the entire field were dark there would be nothing to see: it is merely the back*ground* that is dark. This is very logical reasoning, but the term bright-ground is never used. For consistency, and to help understanding, the term dark-field is used in this book, since it is congruent with bright-field. Incident illumination is often used to mean reflected light or top lighting, but all illumination is incident upon the specimen, either upon the top or on the bottom of the specimen. Epi-illumination is the term for reflected-light illumination. Sometimes dia-illumination is used as a term for transmitted-light illumination (e.g. in Nikon product brochures). The somewhat imprecise term 'dioptre adjustment control' has been retained for convenience. The dioptre is a unit of refractive power expressed in reciprocal metres, whereas the adjustment control compensates for the refractive power of the eye to enable relaxed viewing through the microscope.

5. Mathematical formulae
Formulae are merely precise ways of encapsulating an idea that would otherwise require a long sentence. It may help to ask yourself what is the idea behind the formula. As far as possible, mathematical explanations and formulae have been kept to a minimum, and where possible have been 'quarantined' in their own boxes within the text.

6. Information boxes

Microscopy is a dynamic science, that has evolved through human enquiry and endeavour; the work of a few important individuals has been highlighted to show this and to inform the reader. The box of information on August Köhler was the first written, there being to date very few biographical details in English.

7. References and Internet Resources

Where references are cited, they are given so that you, the reader, can pursue a topic in more depth and/or gain access to further technical aspects of the subject that cannot be included here for reasons of space. They are deliberately fulsome in those chapters that cover fast-evolving subjects, such as light-sheet and super-resolution microscopy. The Key Reading suggestions are papers that, in the author's opinion, are significant and/or contribute extra interest and understanding to the subject of the chapter concerned. The Further Reading is included so that those who wish may broaden their vista of the subject. The decision to include Internet Resources has been made because these are conveniently and immediately available for download. The difficulty with material from the Internet lies in the fact that it is not peer-reviewed or in some cases does not respect copyright and can contain both subtle and gross errors of omission or commission. Furthermore, web pages may become dated. This is not as readily obvious as with printed matter and, of course, URL links may become broken or taken down over time. All links were checked for connectivity during the book production period of June–October 2018.

8. Illustrations

I have tried, as far as possible, throughout this book to include illustrations of microscopes, objectives and other components equally from between the manufacturers of imaging equipment. I am grateful to all those in the microscope trade who have assisted me so willingly. Where company names and brands are given, this information is intended to help scientists who are new to microscopy and who would value initial guidance. Although I may have my own preferences, I have tried to be even-handed, not least because I have friends employed in all the major manufacturing companies, and seek not to cause upset or offence. Where companies and products have been inadvertently omitted, I can only apologise.

Introduction

Presumably you are reading this book because you are interested in light microscopy and are either self-taught, attending a course, responsible for using and maintaining a microscope at work – or all of these things. The author, and those who have supported writing this book, have taught about light microscopes for many years, are passionate about microscopy and wish to help you. That is why this book has been written: to help you understand, set up and use a research light microscope properly.

The aim in writing this book is to *educate* and not merely to *train* you in the use of the light microscope. The distinction is important. Education involves learning about the individual parts of the microscope and how they function, a process inseparable from *understanding* how and why they work as they do, particularly appreciating their limitations – hence the title of this book *Understanding Light Microscopy*. Training, on the other hand, consists only of knowing how to operate the various microscope controls, from switching on the lamp to pressing the digital camera button: procedures set out in the manufacturers' instruction manuals. Education is a journey from instruction to understanding. In one sense it is never complete, although you may stop where you wish.

An important aspect of education is that it is a far more enjoyable and rewarding process than training; being educated in the use of the microscope you will truly become in control of the instrument and empowered in several ways: knowing the 'envelope' of proper operation you will be able to adjust the microscope properly, acquire and record far better images – which is the primary goal. You will also be able recognise and correct faults where they may occur and be able to devise special techniques to suit your own specimens.

These potential goals may be daunting, but each step taken is small. You do not need to know very much physics or mathematics[1] to understand microscopy or to work your way through this book. Essentially, the hard work was done by several geniuses from the late 17th century to the late 19th century, one of these being Ernst Carl Abbe, of whom more later. The author is an image facility manager who started out as a biological sciences technician; he is not a physicist or optician. So, please do not worry; mathematical formulae have been kept to an absolute minimum. Each physical concept and mathematical formula or procedure is explained clearly as they arise.

When we want to see something in more detail, we bring it closer to our eyes. At some point no further detail can be seen or we cannot get sufficiently close to the object. In both cases we use an optical aid to help us distinguish, or resolve, further detail. If we cannot get sufficiently close, we might use a telescope or a pair of binoculars. Where we can get close up to the object, we use a magnifying glass or microscope.

It is commonly thought that the function of the microscope is to magnify, and of course they do magnify. This is the layman's view:

> 'Yes, I have a pair of eyes,' replied Sam, 'and that's just it. If they wos a pair o' patent double million magnifyin' gas microscopes of hextra power, p'raps I might be able to see through a flight o' stairs and a deal door; but bein' only eyes, you see my wision's limited.
>
> [Sam Weller to Sergeant Buzfuz in: *The Posthumous Papers of the Pickwick Club*, chapter 34, page 240; Charles Dickens (1837)]

However, magnifying power is not the principal function of the microscope, which is resolving power – the ability to perceive, or discriminate, fine detail in the object and transfer that to the image. A second function is to provide contrast in the image, which is of particular importance for transparent specimens that would otherwise be invisible. Only when the microscope can provide adequate resolution and contrast will it be useful to magnify the image for comfortable viewing. There is little that the microscope user can do to improve resolution of the image – this property is determined by the objective lens and has to be paid for, though it is commonly made worse by misuse and misadjustment of the microscope. Likewise, magnification is usually fixed by design of the objective and eyepieces (and any intermediate lens system) by the manufacturer. Contrast, on the other hand, can often be improved in simple do-it-yourself ways, which can be devised by the ingenious and informed user.

A microscope is any instrument designed to provide information about the fine details of an object. The term thus includes the single-lens magnifying glass (termed the *simple microscope*) the familiar *compound microscope* which uses an objective lens and an eyepiece, electron microscopes, and also instruments that form their images from a mechanical or electrical interaction between a

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[1] It is entirely possible to understand the underlying principles of microscopy without a formal qualification in physics or mathematics. In his study Einstein kept three portraits: Newton, Maxwell and Faraday. He considered them all to have profoundly underpinned progress in science, physics and technology. However, Faraday was noted for his mathematical inability, and used no complex equations in any of his 450 or so publications.

stylus and the surface of the specimen. In this book we shall confine ourselves to microscopes whose images are formed by the visible part of the electromagnetic spectrum, which we call *light*.

There are many types of light microscope, which differ because of the kinds of specimen they are designed to examine and the techniques used. The traditional microscope is the one generally used for biological work and configured for transmitted light (light that passes through a specimen, rather like light illuminating a stained-glass window in a church). Most explanations in this book will centre on this design, partly because it is so common but also because with the optical components arranged one after the other, it is the easiest to explain and also to understand.

Microscopes designed for use in materials science and also for fluorescence microscopy are configured to examine opaque or fluorescently-stained transparent specimens by reflected light, which illuminates through the objective lens. The objective therefore also acts as its own condenser. These microscopes are generally rather easier to use than the transmitted-light design, because whenever the objective lens is changed, the condenser is also changed appropriately and no major readjustment or centring are required.

Transmitted- and reflected-light microscopes are made in both the conventional upright design in which the specimen is examined from above, and also the inverted form where it is examined from below. Inverted microscopes are particularly useful (and widespread) in biological research laboratories, where they are used to view specimens in water or culture medium. An example would be cell monolayers or tissue explants adherent on a coverslip or grown on the bottom surface of a culture vessel, such as a Petri dish with a coverslip bottom.

In the last thirty years, light microscopy has benefited tremendously from advances in computing, fluorescent markers and laser technology. Fluorescence microscopy has become a ubiquitous technique in biological and medical science in recent years, and most biological microscopes can be adapted to illuminate the specimen with short-wavelength radiation and to observe the emission of longer-wavelength light from fluorescent compounds and proteins stained, or inherent within, the sample.

Most microscopes are designed to operate at high resolution, but a consequence of this is that they have extremely shallow depth of field where the full extent of the object is in focus in the image. Stereomicroscopes adopt a different approach, sacrificing ultimate resolution for increased depth of field. Stereomicroscopes are useful for examining solid objects by reflected light (including fluorescence), although they can be used with transmitted light also. The design essentially uses two microscopes, one for each eye but offset in slightly different directions to produce a three-dimensional effect. In this respect, stereomicroscopes are more like our eyes than the compound microscopes described above and so are particularly suited for educating school children.

These technological advances that heralded the renaissance of the light microscope have come at a price. Unlike other scientific instruments, which will not work unless they are adjusted properly, the light microscope will give an image – however poor – at the flick of a switch, however badly it may be set up. For this reason superficially, practicing microscopy appears simple, but scientific journals are replete with examples of poor quality images. Imaging is more than just 'the bit tacked on the end' of the experiment.

> To correctly capture images using a modern microscope, researchers must have a good grasp of optics, an awareness of the microscope's complexity…such skills can take months or even years to master [which] owing to inexperience or the rush to publish, are all too often squeezed into hours or days. … Inept microscopy, and subsequent analysis, can easily generate results that are misleading or wrong.
>
> [Pearson, H. (2007). 'The Good, the Bad and the Ugly'. *Nature* vol 447 (7141): 138–140, p. 138.]

Modern research microscopes are both very expensive and complex, because viewing anything but the thinnest fluorescent cells and tissues requires optical sectioning to discriminate fine detail within the sample. In order to provide a range of different approaches to detecting and visualising the precise location of fluorescent markers by optical sectioning and kindred techniques such as multi-photon microscopy, several different fluorescence microscopes may routinely be used. Therefore, research institutions and university departments increasingly pool their resources to fund and operate these instruments within centralised facilities.

Image facilities vary enormously and are usually very busy places. Some incorporate both light and electron microscopes together with flow cytometers and high-throughput plate readers; others house light and electron microscopes separately. By their very nature, light microscope facilities have many clients: typical numbers are between 150 and 300 users in any one university or institute. Generally, people who manage imaging facilities have been drawn into the role via a research background as a scientific investigator. Some facilities have several staff to advise about, maintain and operate equipment; others have a sole manager/operator.

If you are reading this book prior to using an imaging facility, it may help to bear in mind that staff, although knowledgeable, may not have as much time as they would wish to help you. Also, there may be greater emphasis placed on image acquisition than subsequent analysis – not least because there is an 'activation energy' associated with the learning curve for new software and your particular application. However, good facility managers should not only be familiar with their equipment but should be also approachable towards their client base.

Image acquisition and analysis is time consuming. The *raison d'être* of this book is to enable you to set up your microscope and collect good images competently and efficiently, unaided.

Treating the microscope like a powerful holiday-slide viewer ignores the fact that with the microscope, we are using light for 'something it wasn't designed for' – studying little things whose dimensions are close to the wavelength of the illuminating light itself. When we use a microscope we do not look at the object directly, but at an *image* of it. Therefore – like a painting or a sculpture – the microscopical image is only a *representation* of the object, and that image can be altered by the quality of the microscope, its objective lens and how light interacts with the specimen.

The prime purpose of this book is to guide you in avoiding these pitfalls whether you are beginning in microscopy or have been a microscope user for some time and wish to learn more. Lack of instruction is nothing new. Henry Baker's *The Microscope made Easy* (1742) had this advice in chapter 15, entitled 'Cautions in Viewing Objects', which is still pertinent. Baker warns us thus: