Fundamentals of light microscopy

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PREFACE

Despite enormous advances in electron microscopy (most recently in the scanning of surface structure) the light microscope is far from obsolete, and new applications are still being invented. The most striking of these have been in the biomedical area, where fluorescence microscopy in particular has opened up whole new fields of research. Phase-contrast and the newer interference-contrast microscopy remain unrivalled for observing living cells, while quantitative interference techniques and polarizing microscopy find application in the industrial field as well. The light microscope is probably the biophysical tool most widely used by non-physicists.

The electron microscope is essential for visualizing detail at the molecular level, but it cannot match the ability of the light microscope to focus at different levels within a three-dimensional object; nor can it offer the range of specific staining techniques available to the light microscopist. In biology the data from all kinds of microscopy are needed to study different levels of organization. There are, however, inherent problems of limited resolution in light microscopy, and a possibility of optical artifacts, that are just as real today as they were two centuries ago (Frontispiece). The aim of this book is to explain to the practical microscopist in mainly non-mathematical terms the basic principles underlying each branch of light microscopy, so that he or she can get the best out of an instrument and avoid misleading results. Some mathematical treatments have been collected in the Appendix, but these should be regarded as strictly optional.

The book should also help the research worker in choosing what is often a fairly expensive piece of apparatus. It is designed particularly for biomedical students, and for all those preparing for professional examinations in microscopy. It arose out of a course of lectures given for some years past in King's College. Our students have mainly been undergraduates, but the course has always been open to postgraduate students and research workers from other institutions. We have therefore had the benefit of interacting with enthusiasts from a wide range of backgrounds, some of whom had never studied physics before and needed a concise but intellectually-satisfying explanation of the basic principles. At the same

time, many undergraduates who had studied physics at 'A' level were glad to be reminded of what they had once learnt but since forgotten.

A few topics have deliberately been excluded because I believe them to be better left to the specialized textbooks that are available; they include staining techniques, micrometry, stereology, and the use of various scanning devices for making quantitative measurements. Scanners rely on the integration of the light transmitted by a specimen when traversed by a 'flying spot'. Apart from this they involve no new principles, and have the same basic optical system as an ordinary microscope. Their high cost means that only a research worker is likely to have access to them, and the manufacturers of such devices provide adequate instructions. I have, however, included a brief discussion of photomicrography; the arrangements for taking pictures vary between microscopes, but the basic principles are the same as in any application of photography.

For help in providing material and making suggestions I am particularly indebted to the late Howard Davies, who was instrumental in setting up the original microscopy course, and to Tom Cavalier-Smith who currently organizes it. I am also grateful to many colleagues and others who have read and criticized the manuscript. The half-tone illustrations owe much to the skill of Zoltan Gabor, and originals were kindly provided by Donald Olins (Frontispiece), Michael Dickens (Fig. 13), John Couch and Ed O'Brien (Fig. 21), Derek Back and Howard Davies (Figs 23 and 26), Clive Thomas (Fig. 30), and John Couchman (Fig. 43). The Frontispiece was previously reproduced in Ts'o, P.O.P. (ed.) (1977) *The molecular biology of the mammalian genetic apparatus, Vol. I* (North-Holland, Amsterdam).

CONTENTS

1 Microscope alignment

We shall see in later chapters why there is a theoretical limit to the ability of a light microscope to see fine detail, or to distinguish closely-spaced objects from each other. Many biological objects are of a size close to this resolution limit, which is defined as the smallest detectable separation of two point objects. It is clearly important that the instrument is set up to approach the limit as closely as possible. With good design this can be achieved much more readily than for an electron microscope, whose resolution is often far short of its theoretical limit. A student who has used only the preset microscopes provided in a practical class may not appreciate what a difference it makes to have the optical system properly aligned. In particular, there are theoretical grounds for the belief that the form of the *illumination* affects the resolution. Here we consider how this and other equally important factors are optimized when setting up a microscope. The further alignment of specialized instruments such as the phase microscope is discussed in later chapters.

Basic geometrical optics

This section is for those who would like to be reminded of the fundamental properties of lenses. Any microscope can be considered as equivalent to only two simple, converging (magnifying) lenses, representing the objective and the eyepiece. The condenser is equivalent to a third lens, and a fourth is used to achieve *Köhler illumination* (see next section). In practice, of course, each component in the system contains many optical elements, but this does not concern us now.

A *converging* lens has two *foci* (F_1 and F_2 in Fig. 1(a)) which are the meeting points of parallel rays of light striking the opposite side of the lens. The *focal length*, f, is the distance from the centre of the lens to either of the two foci. Fig. 1(a) also illustrates why the *depth of focus* (related to the distance over which the rays converge more or less to a point) is greater when the lens aperture is restricted by a *stop*; on either side of F_2 the light forms a blurred circle instead of a point image, but with a stop in position

one can move further away from F_2 before the effect is noticeable. This is why there is no need to adjust the focus of a cheap camera whose aperture is permanently restricted.

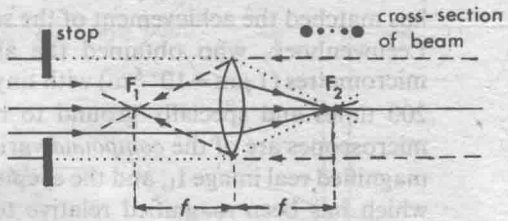
A converging lens can produce two kinds of image. A *real image* (one that can be formed on a screen or a photographic film) arises when the object is placed so that its distance from the lens is greater than the focal length. There are mathematical formulae (Fig. 1(b)) for predicting both the position and the magnification of the image in terms of u (distance of object from lens), v (distance of image from lens), and f. However, there is also a very simple graphical method of determining them. From a point on the object draw two rays, one of which travels parallel to the lens axis OL, while the other passes through the centre of the lens. The first ray must bend so as to pass through the focus at point F_2 on the other side of the lens, while the second ray is not changed in direction because the centre of the lens behaves like a parallel-sided block of glass. The image is formed, upside down, where these two rays intersect.

For a given depth of focus in the image there will be a proportional *depth of field* in the object, for which a tolerably-sharp image is obtained. Low-power microscope objectives have in general a greater depth of field than high-power ones.

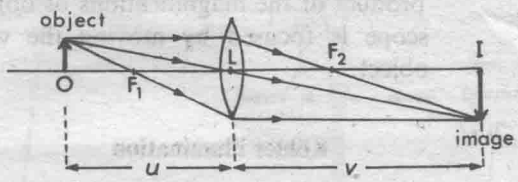
A *virtual image* (Fig. 1(c)) is formed by a converging lens when the object is placed so that its distance from the centre of the lens is *less* than the focal length. Rays drawn as before will no longer converge to a point; however, a detector (such as the eye or a camera) that incorporates an additional converging lens can form a real image. The image *appears* to be situated on the same side of the lens as the object, and in this case it is not inverted. When viewing by eye, the distance from eye to image must not be less than the minimum distance for comfortable viewing (about 250 mm). For completely relaxed eyes, the virtual image can be 'at infinity' as in Fig. 1(d); this arises when the object lies exactly at the focus of the lens. The eye regards it as a distant object (such as the sun) whose size can only be expressed in terms of an angle β (beta) between the rays reaching the eye from the extreme edges of the object.

This leads to a convention which defines the magnification of a virtual image, and which is commonly used for microscope eyepieces. The magnification is taken as the ratio of the angles β and α (alpha) made at the eye by the image, and by the object when viewed directly at a distance a of about 250 mm. Incidentally, some people find it impossible to relax their eyes completely when using a microscope, and they focus it to form a virtual image quite close to the eye. This can lead to problems when setting up for photography, unless one uses an attachment to form a real image on a ground-glass screen.

An ordinary magnifying glass forms a virtual image, but there is a



(a) focal length and depth of focus

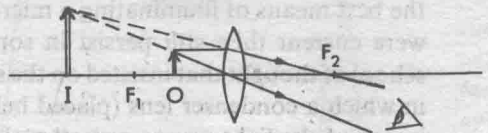


(b) real image formation

$F_1L = LF_2 = f$

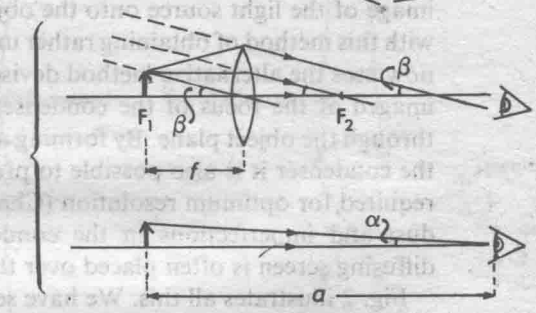
$$\frac{1}{u} + \frac{1}{v} = \frac{1}{f}$$

magnification $M = \frac{v}{u}$



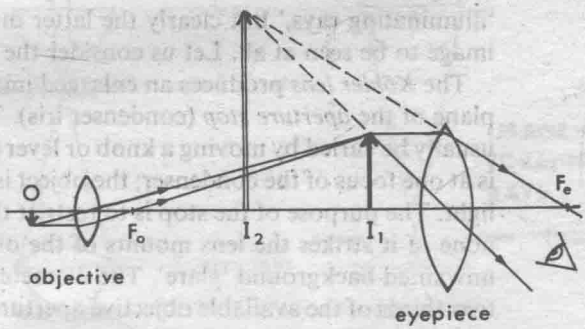
(c) virtual image formation

$$\frac{1}{u} - \frac{1}{v} = \frac{1}{f}$$



(d) object at focus, image at infinity

$$M = \frac{\beta}{\alpha} = \frac{a}{f}$$



(e) compound microscope (schematic only; I_2 should in practice be at infinity)

Fig. 1. Properties of converging lenses.

practical limit to the magnification obtainable. Nobody in modern times has matched the achievement of the seventeenth century microscopist van Leeuwenhoek, who obtained the astonishing resolution of about 1.5 micrometres (1 μm = 10^{-6} m) with tiny single lenses, magnifying more than 200 times and specially ground to reduce the aberrations. All modern microscopes are of the *compound* variety (Fig. 1(e)); the objective forms a magnified real image I_1, and the eyepiece generally forms a virtual image I_2 which has been magnified relative to I_1. The total magnification is the product of the magnifications of objective and eyepiece, and the microscope is focused by moving the whole combination relative to the object.

Köhler illumination

There was a violent controversy which lasted until the 1930s over the best means of illuminating a microscope, and the misconceptions that were current then still persist in some modern textbooks. There was a school of thought that insisted on the special virtue of 'critical illumination' in which a condenser lens (placed below the object) is used to project an image of the light source onto the object plane. There is, however, a risk with this method of obtaining rather uneven illumination. Nearly everyone now uses the alternative method devised by Köhler, in which the source is imaged at the focus of the condenser to give parallel (unfocused) light through the object plane. By forming a magnified image of the lamp below the condenser it is also possible to provide the wide cone of illumination required for optimum resolution (Chapter 4), and to reduce the effects of dust and imperfections in the condenser. As a further precaution, a diffusing screen is often placed over the source.

Fig. 2 illustrates all this. We have separated 'image-forming rays' from 'illuminating rays,' but clearly the latter must enter the eye pupil for the image to be seen at all. Let us consider the illuminating rays first.

The *Köhler lens* produces an enlarged image of the lamp filament in the plane of the *aperture stop* (condenser iris). This stop, whose diameter can usually be varied by moving a knob or lever projecting from the condenser, is at one focus of the condenser; the object is evenly illuminated by parallel light. The purpose of the stop is to restrict the cone of illumination so that none of it strikes the lens mounts of the objective; this would lead to an unwanted background 'glare'. The diameter is normally set so that about two-thirds of the available objective aperture is filled with light, though for special purposes it may be further reduced. The objective forms another image of the source (and one of the aperture stop) in its *back focal plane*, and the eyepiece causes the rays to converge again towards the pupil of the eye. In order to test whether the lamp is properly aligned one can use a

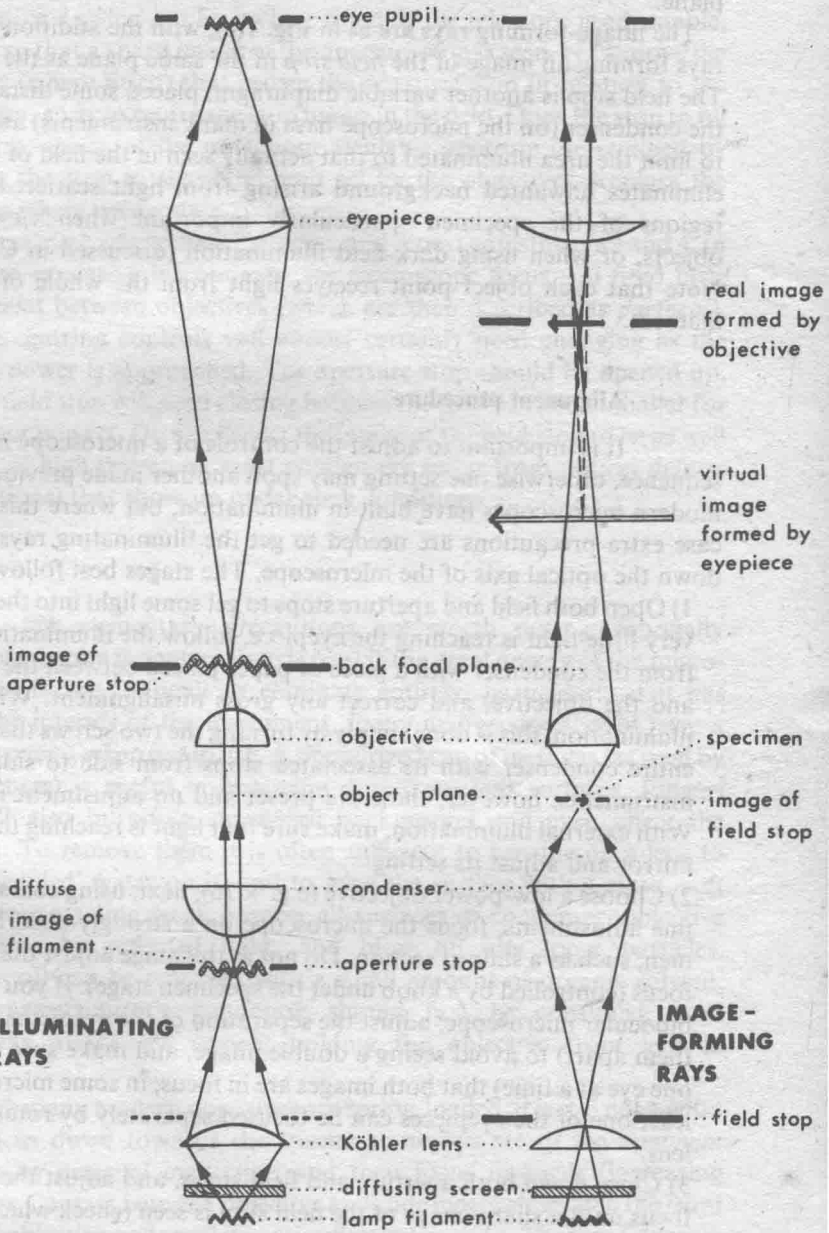


Fig. 2. Köhler illumination.

phase telescope (or a swing-out *Bertrand lens* if fitted) to view the back focal plane.

The image-forming rays are as in Fig. 1(e), with the addition of a set of rays forming an image of the *field stop* in the same plane as the specimen. The field stop is another variable diaphragm, placed some distance below the condenser (on the microscope base in many instruments) and it serves to limit the area illuminated to that actually seen in the field of view. This eliminates unwanted background arising from light scattered by other regions of the specimen – particularly important when viewing faint objects, or when using dark-field illumination (discussed in Chapter 5). Note that each object point receives light from the whole of the lamp filament.

Alignment procedure

It is important to adjust the controls of a microscope in the right sequence, otherwise one setting may spoil another made previously. Most modern microscopes have built-in illumination, but where this is not the case extra precautions are needed to get the illuminating rays travelling down the optical axis of the microscope. The stages best followed are:

1) Open both field and aperture stops to get some light into the system. If very little light is reaching the eyepiece, follow the illumination coming from the condenser with a piece of paper placed between the condenser and the objective, and correct any gross misalignment. With built-in illumination, this is done simply by turning the two screws that move the entire condenser with its associated stops from side to side; in some instruments, however, these are preset and no adjustment is possible. With external illumination, make sure that light is reaching the substage mirror and adjust its setting.

2) Choose a low-power objective (e.g. × 16); next, using coarse and then fine adjustments, focus the microscope on a strongly-absorbing specimen, such as a stained section. Do not at this stage adjust the condenser focus (controlled by a knob under the specimen stage). If you are using a binocular microscope, adjust the separation of the eyepieces (by sliding them apart) to avoid seeing a double image, and make sure (by closing one eye at a time) that both images are in focus; in some microscopes, at least one of the eyepieces can be focused separately by rotating its top lens.

3) Close down both aperture and field stops, and adjust the condenser focus until a sharp image of the field stop is seen (check which stop you are focused on by varying their diameters). Next centre the condenser (where possible) so that the field-stop image is in the middle of the field of view, and open the field stop again to just fill the field.

4) Where a bright-field microscope is being used, close down the aperture stop attached to the condenser. Insert a phase telescope in place of the eyepiece, or a swing-out lens where fitted. If the telescope is adjustable, focus it so that a sharp image of the aperture stop is seen. Now move the controls (where fitted) that centre the aperture stop in relation to the condenser, so as to centre the stop image in the field. Open the stop to fill about two-thirds of the maximum available aperture (determined by opening the stop to show the limit set by the objective). Replace the eyepiece where necessary.

5) Change to a higher power if needed, and repeat adjustments 3 and 4. In the more expensive instruments, the microscope focus will need little adjustment between objectives (which are then described as *parfocal*), but the centring controls will almost certainly need changing as the highest power is approached. The aperture stop should be opened up, but the field stop will need closing because the field of view is smaller for the higher powers. Do not expect the image of the field stop to be as well defined at high power, as most condensers suffer from optical defects (aberrations) that show up under such conditions.

A few elementary precautions are worth noting, especially where photography is contemplated. *Dust* is the great enemy of the microscopist and is very difficult to eliminate entirely, particularly if it has got into the interior of the instrument. Every microscope should have a dust cover over it when not in use. A pressurized can of inert gas (as used by photographers) is useful in removing dust from lens surfaces. Fingermarks will also introduce unwanted background and even affect the resolution. To remove them it is often sufficient to breathe on a lens to deposit 'distilled' water on it, and to wipe the surface with a clean, soft tissue. Before starting work, inspect all surfaces of condenser, objective and eyepiece by reflected light, and blow off any loose particles. Immersion oil can be removed with a small amount of organic solvent, but the manufacturer's handbook should first be consulted; even alcohol may attack the cement holding the objective front lens in place.

Although many modern objectives are spring-loaded, it is still not a good idea to focus down towards the specimen; always set to the *minimum* separation by external inspection and then focus upwards (increasing separation). Contact between objective and slide may well scratch the front lens of the objective and spoil its anti-reflection coating. The same dangers exist for any component or accessory—always put it in a safe place rather than simply leaving it on the bench.

Questions to test your understanding (*answers follow Appendix*)

1. If given only a magnifying glass, a piece of card and a ruler, how could you estimate the focal length of the lens?
2. With the equipment listed in question 1, how could you define the magnification of the lens?
3. When a magnifying glass is used in conjunction with the relaxed eye and an object is placed at the focus of the lens, where is the image formed?
4. If a transparency is illuminated from behind and an image of it projected onto a screen by a lens, what will happen if the illuminating lamp is moved to one side – will the image move, or not?
5. If light is scattered from regions of an object outside the field of view, will spurious images ever be formed? If no spurious images are formed, is the scattered light in any way detrimental?

2 Properties of light

Basic principles – why bother to learn them?

We first have to consider the wave properties of light. Wave theory is essential in explaining how transparent objects are made visible by phase or interference techniques; why there is a limit to the detail that can be seen; how an instrument's performance is tested; and how quantitative measurements are made with polarizing and interference microscopes. Only qualitative staining techniques, where light is simply absorbed by the object, require no further understanding of the process involved – and even then it is a good idea to know enough about optics to optimize the illumination. Staining is not, however, usable when we need to visualize transparent objects without subjecting them to chemical insults, nor can it in general be used for determining molecular orientation or the dry mass of an object.

There is a further bonus for those who master the basic principles of physical optics: the theory applies directly to both X-ray diffraction and optical diffraction, now a popular tool for analyzing electron micrographs. Although this book deals only briefly with such techniques, it should give the reader enough insight to understand how the specialists obtain their information, and how reliable it is.

Waves and particles

It is a long-standing paradox of physics that light waves must sometimes be treated as if they consisted of particles in order to explain their behaviour, while electrons have sometimes to be regarded as waves. However, waves and moving particles have in common the property of carrying *energy* from the source to the observer. The eye is sensitive only to changes in brightness and in colour, so in microscope images the brightness or *intensity* is made to vary across the field of view. As we shall see, many biological objects do not in themselves generate intensity changes, and a special attachment (as in the phase microscope) must be used to do this by making visible the variations in refractive index. The same problem arises in photomicrography.

Properties of waves

A wave is the result of propagating a disturbance from a source, as in the ripples made by a stone dropped in a pond. Energy from the source (for instance, thermal energy in the atoms of a lamp filament) is fed into the waves. In the case of monochromatic waves like those generated by a discharge lamp or a laser, the wave responsible for a given colour (e.g. the intense green line of a mercury lamp) is characterized by a *frequency*, measured in Hertz (cycles per second), which is invariant; an observer anywhere in the path of the wave will measure the same frequency, and the frequency determines the perceived colour of the light. The rule only breaks down where there is rapid relative motion of source and observer, a situation that does not concern us here.

In the generation of a sound wave, some kind of surface vibrates, causing variations in air pressure which travel out from the source. For light waves from a hot filament or a discharge lamp, each atom acts as an independent source – only in a laser do they act in unison. In addition, the energy given out by each atom comes in bursts (quanta), and the timing of the start of each burst is independent of that of the last one. We shall see the relevance of this later.

A light wave, like any electromagnetic wave, is propagated as a fluctuation in electric and magnetic *fields*. An example of a static electric field is shown in Fig. 3, where parallel plates are connected to a battery. Note that the field, unlike the pressure variation in a sound wave, is directional and has a sign as well as a magnitude which can be measured with a suitable instrument; reversing the battery terminals will reverse the direction of the field. In a propagated wave (also shown in Fig. 3) there is a periodic oscillation in electric field along the direction of propagation, and

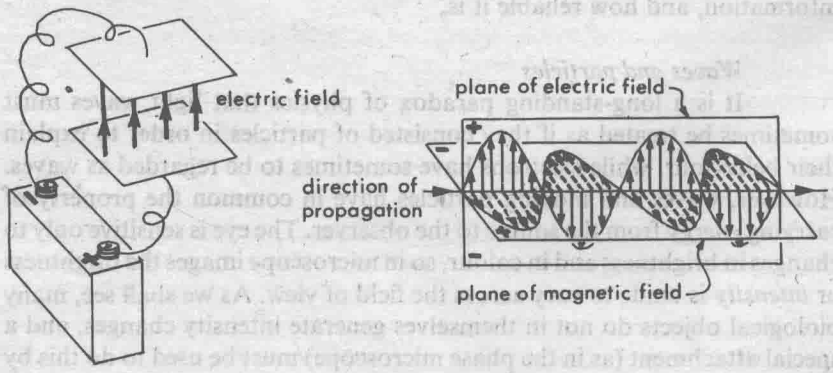


Fig. 3. An electric field and an electromagnetic wave.