From: Sanderson, J ***Understanding Light Microscopy*** to be published by John Wiley & Sons, ISBN: 978-0470973752

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 maintaning 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 inseperable 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 recogise 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 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.

[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.

It is commonly thought that the fuction 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.'
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> [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 which form their images from a mechanical or electrical interaction between a 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 which 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 are 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 stains 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 which heralded the rennaisance 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.'
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> [Pearson, H (2007) The Good, the Bad and the Ugly *Nature* vol 447 (7141): 138-140, page 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 micoscopy, 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.

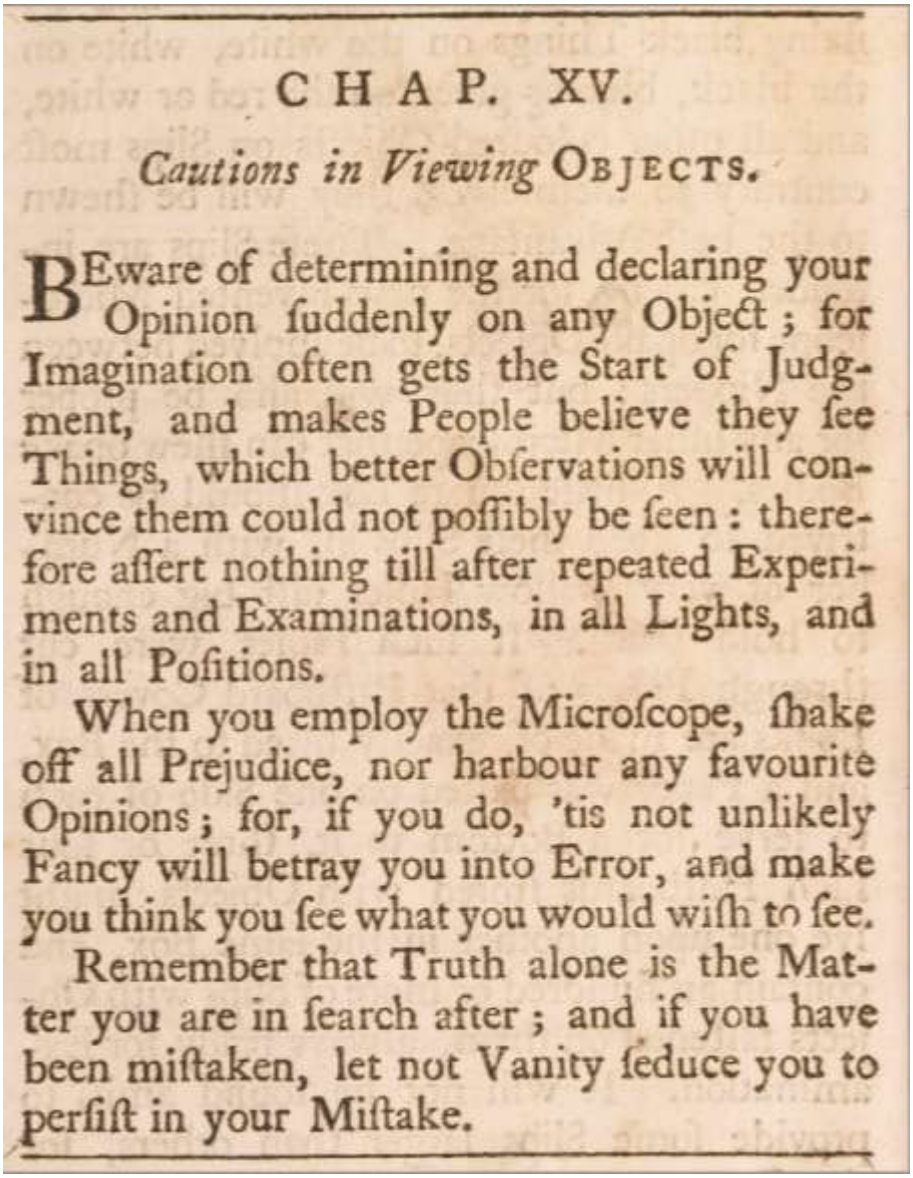
Image facilities vary enormously and are usually very busy places. Some incorporate both light and electron microscopes together with flow cytometers and high-throughput plate readers; others house light and electron microscopes separately. By their very nature, light microscope facilities have many clients: typical numbers are between 150 – 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 approachable towards their client base.

Image acquisition and analysis is time-consuming. The *raison d'etre* 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:



CHAP. XV.

Cautions in Viewing OBJECTS.

BEware of determining and declaring your Opinion ſuddenly on any Object; for Imagination often gets the Start of Judgment, and makes People believe they ſee Things, which better Obſervations will convince them could not poſſibly be ſeen: therefore aſſert nothing till after repeated Experiments and Examinations, in all Lights, and in all Poſitions.

When you employ the Microſcope, ſhake off all Prejudice, nor harbour any favourite Opinions; for, if you do, 'tis not unlikely Fancy will betray you into Error, and make you think you ſee what you would wiſh to ſee.

Remember that Truth alone is the Matter you are in ſearch after; and if you have been miſtaken, let not Vanity ſeduce you to perſiſt in your Miſtake.

Reference: Baker, H (1742) *The Microscope made Easy*, chapter 15, pages 62-63

Most books and courses start teaching microscopy by explaining some optics prior to evaluating the instrument itself. The layout of this bok is broadly similar, but it is important not to forget the specimen, the object of study in the first place, whose fine detail we wish to resolve, to study and to understand. It is the interaction of light with the specimen that gives rise to any detectable signal. If this specimen is living, is marked with fluorescent probes, and is being studied over time, then the constraints on collecting suitable images are different, and intrinsically more challenging, than for fixed immobile samples.

Chapters 1-3 in this book cover the need for microscopes, some basic optical principles and a treatment of ray optics. Chapters 4 & 5 explain how different microscopes differ in design according to their function, and how to apply ergonomics to the safe use of the microscope. Chapters 6-8 cover the objective and other components of the microscope, as well as a treatment of optical aberrations. Chapter 9 explains how to adjust and set up a microscope properly. Chapter 10 tells you about diffraction and wave optics: how images are formed. Chapters 11 & 12

explain how to generate contrast with both transmitted- and reflected-light. Chapters 13 & 14 explain the theoretical fundamentals of polarised-light and how to apply these in a practical manner. Chapters 15-26 cover all aspects of the theoretical and practical application of fluorescence microscopy, including optical sectioning, colocalisation and super-resolution imaging. Chapter 27 advises on which fluorescence technique to use, as well as giving advice on using an imaging facility. Chapters, 28 & 29 discuss sample preparation for biological and materials specimens respectively, while the final two chapters, 30 & 31, cover how best to record the image.

As stated at the outset, the aim is not merely to instruct, but to help you understand. Where applicable, explanations are underpinned by a few simple experiments that you can try yourself to aid your understanding. Illustrated text boxes are used to emphasise key facts or convey points of interest. The didactic approach comes from experience teaching students, those attending the Royal Microscopical Society's Summer Schools in Light Microscopy, and also from managing light microscope imaging facilities over several years. Myself and my colleagues acknowledge the debt from students for their questions and feedback about our teaching. We know that many students come to us with misconceptions which they are honest and humble enough to admit to, and so ask for help. We admire them.

Jeremy Sanderson
Oxford, October 2016