The Light Microscope

Jeremy Sanderson
BIOIMAGING FACILITY MANAGER, MRC HARWELL

HUMANS ARE VISUAL, AND THE LIGHT MICROSCOPE IS THE ICON OF SCIENCE. YET FEW CAN ADJUST A MICROSCOPE PROPERLY TO GIVE A GOOD IMAGE, OR REALISE HOW SOPHISTICATED MODERN LIGHT MICROSCOPES HAVE BECOME. THIS ARTICLE DESCRIBES THE DEVELOPMENT OF THE LIGHT MICROSCOPE, HOW TO SET ONE UP, AND HOW MODERN LIGHT MICROSCOPE TECHNIQUES ARE AT THE FOREFRONT OF SCIENTIFIC AND MEDICAL DISCOVERY.

The word 'lens' comes from the Latin for lentil whose shape describes a double-convex lens. Whilst the precise origins of the light microscope are disputed and lost in history, microscopes were being effectively used in the 17th century by Antoni van Leeuwenhoek and by Robert Hooke. Leeuwenhoek made his own single lens microscopes (Figure 1). Since these were of high magnification, they would necessarily possess a very short focal length and have been rather hard to use. Instead, Hooke used a compound microscope (Figure 2) which has many parts in common with a modern entry-level light microscope (Figure 3), that we might use today.

Most people think that the purpose of the microscope is to magnify. This is not so. The chief purpose of any microscope is to resolve fine detail. The second requirement is for sufficient contrast to discriminate these resolved details in the image. Magnification is the third and least important requirement; its only function is to present details resolved by the microscope objective in the image at a size that our eyes can see.

Two significant steps were taken in the 19th century that perfected the design of the light microscope, making possible concomitant advances in science and medicine. Firstly, by 1830 Joseph Lister had combined a plano-concave element of flint glass cemented to a plano-convex element of crown glass. This was a great improvement, giving clear, sharp images vastly reducing the effects of blurring from spherical aberration and colour fringing from chromatic aberration. All lenses suffer from optical aberrations to one degree or another, but by careful design it is possible to minimise these. Because objectives are expensive to manufacture, they are offered in three grades: achromats, semi-apochromats (commonly referred to as 'fluorites') and the best corrected of all: apochromat objectives.

The second significant discovery occurred in Germany in 1873. Whilst opticians understood that aberrations could be minimised by combining lens elements, no-one understood

Pic credit: Zeiss

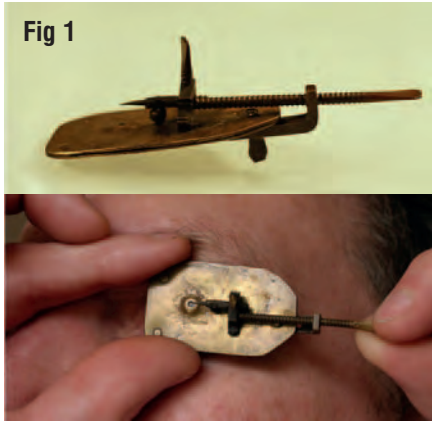


Fig 1

Fig 2

The early microscopes of Leeuwenhoek and Hooke. Many of the components found on a modern microscope (light, condenser, objective, bodytube, eyepiece) can be seen on Hooke's microscope

how to design objectives of sufficient resolving power. Lens elements were combined empirically, hoping for a good result. Consequently, Carl Zeiss very nearly went out of business. He hired a young lecturer, Ernst Abbe, to calculate the theoretical basis of image formation and place microscope manufacture on a rational footing. This single technical advance underpins all modern microscopy, even the advances of the 21st century in 'super-resolution' techniques.

When an object is illuminated, light rays are diffracted by the fine structures in that object. A lens can collect these rays and form an image by refraction. The resolving power of a microscope is determined by the wavelength of the illuminating radiation and by the ability of the objective lens to collect these diffracted rays to form an image. Shorter wavelength blue light diffracts less than longer wavelength red light. The finer details in the image are encoded by the higher order diffracted rays, so the greater the aperture of the objective and the shorter the wavelength of the illuminating radiation, the finer the detail that will be resolved in the image. These relations may be expressed in the following equation: $d = \lambda/2NA$, where d is the size of the resolved detail, λ is the wavelength and NA the numerical aperture of the objective. It was Abbe who coined the term 'numerical aperture', the single most important

Fig 3

A modern entry-level polarising microscope with digital integral camera. This equates broadly with Hooke's compound microscope. The lighting is now integral within the base of the stand. The microscope can be used both in reflected-light mode (as for Hooke's microscope) or in transmitted-light mode

quality of a microscope objective, since it determines the resolving power and light-gathering capacity of the objective. Numerical aperture values can be directly compared: an objective of NA 0.8 (regardless of magnifying power) will resolve twice as much detail in the image as an objective of NA 0.4.

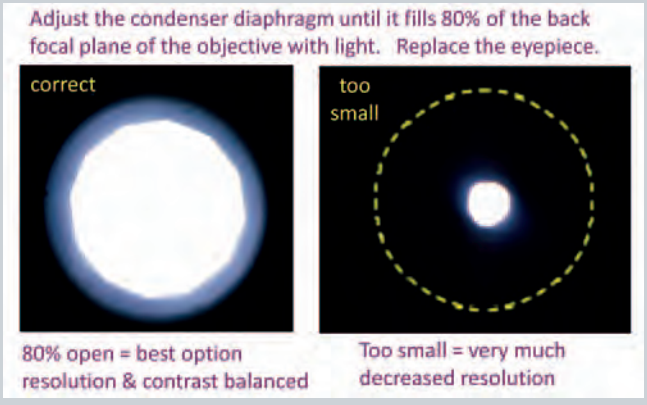
The closing years of the 19th century saw a final significant development still in use today - the introduction of a reliable method of adjusting the microscope to exploit the full aperture of the objective - Köhler illumination. In the 1890s August Köhler was a PhD student who wished to photograph molluscs. Prior to the development of the light bulb, microscopists had to rely upon good natural lighting from the sun or else use an oil or gas lamp. All these methods had their drawbacks: the sun could be obscured by heavy cloud; oil and gas were awkward to use and of low luminous flux. However, the filament of an electric light is small, and showed up on the image. Köhler introduced a lamp collector lens to spread the light in the specimen plane evenly across the field of view. As a consequence, there are two sets of inter-digitated conjugate planes: the imaging set (containing the illuminated field diaphragm) and the illuminating set (containing the condenser diaphragm). Therefore a protocol (see Box) has to be followed to adjust the microscope properly to form a high-quality image. Otherwise it is very easy to accept a poor image without realising that the microscope is not being used to its full potential: a cheap child's toy microscope might as well be substituted for a high-end research microscope costing thousands.

After the Second World War, much effort went into the development of both the transmission and scanning electron microscopes in an effort to improve resolving power, to see ever finer detail.

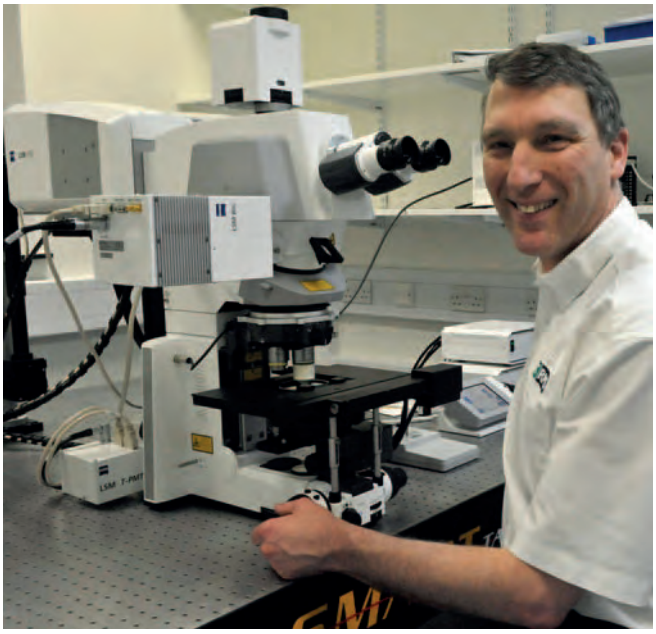
Setting up the light microscope

There are four steps to adjusting a microscope properly for 'Köhler illumination':

1. Focus the specimen upon the stage, taking care not to run the objective into the slide or sample. It is best to bring the objective within the point of focus whilst looking side-on, then focus using the eyepieces.
2. Close down the *illuminated field diaphragm* on the base of the microscope stand to its smallest extent. Adjust the height of the condenser to show the leaves of this iris diaphram in sharp focus. Open the diaphragm to just fill the field of view evenly with light, which will depend on the magnification of the objective used. Centre the field diaphragm if necessary.
3. Open the *condenser diaphragm* to fill the back aperture of the objective with light: all the diffracted rays captured by the objective can then contribute to image formation. Do this by removing an eyepiece and looking at the back focal plane of the objective. To improve contrast in the image, close this diaphragm to about 80% of the overall diameter seen. The exact adjustment will depend on the NA of the objective and the contrast in the image. Replace the eyepiece.



4. Finally adjust the dioptre controls of the eyepieces for comfortable strain-free viewing. Focus using the highest dry objective. Without altering the focus, swing in the lowest power objective. Adjust the eyepiece dioptre control(s) to give a sharp image.
5. Swing in the required objective, check the field diaphragm and condenser diaphram settings, and proceed.



Jeremy Sanderson pictured with modern 21st century two-photon microscope with laser-scanning confocal capability. A modern research microscope such as this is multi-functional with integrated software control for consistency, reproducibility and ease-of-use. Prices vary according to the specification ordered, but can range between £100-500k

By the 1970s many considered that the future of microscopy lay with the electron microscope, the light microscope facing only a limited future. Nevertheless, the light microscope still has many advantages over the electron microscope. The lenses of electron microscopes have very low numerical aperture; electron microscopy demands very intensive specimen preparation techniques; the images cannot be seen in colour, and the vacuum required for electrons to travel within the microscope precludes the examination of living specimens.

Realisation of the power of fluorescence microscopy (eg. see the article on fluorescent probes on page 55 of the autumn 2014 issue) together with the advent of the laser, powerful computers and electronic detectors for digital imaging, has led to the renaissance of the light microscope. The newest advances in physics and optics are helping to close the gap between the limit of resolving power of the light microscope and electron microscopy. These so-called 'super-resolution' techniques such as STED, SIM, PALM and STORM are able to circumvent Abbe's resolution limit. Scientists are beginning to use the power and versatility of modern light microscopes (see author photograph) to explore, in-situ and in real-time, how proteins and other cellular components function and interact within their natural environment.

For those wishing to learn more about light microscopy, training courses are available. The Royal Microscopical Society offers courses on introductory principles and also on confocal microscopy. Details can be found on the RMS website (http://www.rms.org.uk).

An advanced course sponsored by the European Molecular Biology Organisation (EMBO) is held every spring at the Marine Biological Association, Plymouth (http://www.mba.ac.uk/embo-course).

External courses are also offered by individual imaging facility staff; the author runs a practical introductory course to light microscopy and confocal microscopy at his own institution (http://www.har.mrc.ac.uk/training-courses).

Email: training@har.mrc.ac.uk