SETTING UP THE MICROSCOPE part 2

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Once the lighting system has been correctly set up (Moss, 2000) the resolution and initial magnification of the subject is achieved by the objective and depends on the quality of that objective. Of course the eyepiece effects a further magnification but it cannot improve the resolution achieved by the objective which can justifiably be described as the most important component of the microscope (Bradbury & Bracegirdle, 1998). Objectives commonly range from X4 to X100. Modern microscopes have a nose piece typically taking four objectives which can be rotated into position as required. A popular combination is X5, X10, X40 and X100, the first three being dry and the last an oil-immersion objective. Ideally these should be parfocal, i.e. when one is in focus the others will be also as they are rotated into position; most affordable microscopes are only approximately parfocal.The higher the magnification the closer the bottom of the objective will be to the slide under study, so it is wise to start at the lowest magnification, as this can be confidently brought into focus without any danger of damaging the slide or objective!

Thereafter, objectives of increasing magnification should only need fine focussing if they are reasonably parfocal.

There will usually be separate coarse and fine focus controls. In older microscopes focussing was achieved by moving the tube bearing the objectives and eyepiece. In most modern microscopes it is the stage with the subject to be studied which is moved. The coarse focus will usually operate continuously between the mechanical stops which limit the movement of either stage or tube. The fine focus may also have a limited range. For most purposes it is worth presetting the fine focus control roughly in the middle of its range giving flexibility once the subject is brought into approximate focus. It is, in my experience, useful to be continuously fiddling with the fine focus when studying detail. One sometimes spots some detail as it comes into and out of focus which might otherwise be missed, a bit like catching sight of something moving out of the corner of one's eye. It is essential to know the direction the objective is moving (relative to the slide) as you use the fine focus if you want to know which structures lie above or below others on the slide.

As well as magnification a second property often marked on the objective is the numerical aperture (NA). For any given magnification the higher the NA the better the resolving power of the objective. This parameter is given by the relationship: $NA = n \sin\alpha$, where n is the refractive index of the medium between the slide and objective ($n = 1$ when this is air) and α is the half angle of the maximum cone of light accepted by the objective (see Fig.1). Clearly in air the numerical aperture cannot be greater than 1.0 and in practice the very best plan apochromat X40 objective could have NA = 0.95 implying a value for α of around 72°.

There are various terms used to describe objectives, based on the level of correction of spherical and chromatic aberration inherent in a simple lens. Achromats have been corrected for two wavelengths, in the red and blue parts of the spectrum, so that the light of these two wavelengths is brought to a common focus along the optical axis.

There may still be a fringe of colour around a subject being studied but it is not usually a nuisance during routine work and most of us will be working with achromats.

Apochromatic objectives have been corrected for three wavelengths (red, green and blue) allowing much higher numerical apertures, and hence greater resolution, at a given magnification. A plan apochromat has been further designed to give a flat field over most, if not all, of the field of view. There is an intermediate level of correction known as a semi-apochromat (which was first referred to as a fluorite). Thus, at best, a X40 objective might have NA = 0.65 for an achromat, NA = 0.70 for a semi-apochromat and NA = 0.95 for a plan apochromat but, my goodness, you have to pay a lot of money for that resolution with a dry objective. An oil-immersion achromat of NA = 1.25 may have six elements in the lens system but an oil plan apochromat of NA = 1.4 can have as many as twelve and this does not mean that it will only cost twice as much!

To appreciate why we need oil immersion to achieve increased resolution as well as to work at higher real magnifications it is necessary to consider, not the angle of light entering the objective from the top of the cover glass, but what limits the angle of the cone of light passing through the cover glass (Fig. 2). The refractive index (n) is the ratio of the sines of the angle of incidence and the angle of refraction. For a glass (n = 1.515) to air surface the largest possible value for this angle will be 41.3° which would give an angle of refraction of 90°. Any rays of light leaving the subject at a greater angle cannot enter the objective however good it is! This limitation is overcome by introducing an oil of the same refractive index as glass between the cover slip and the bottom face of the objective thus increasing the amount of light (= information) that can be collected from the subject (Fig. 3).

The X100 objective is always an oil immersion lens and its NA may range from 1.25 for an achromat to 1.4 for a plan apochromat. Some objectives of lower magnification are designed as water immersion lenses and I have such an objective of unknown make X63, NA = 0.85, marked WI. It is also possible (at a price) to obtain 'high dry' objectives also X63 but requiring neither oil nor water.

Most of the study of fungi can be carried out with dry objectives but X100 oil immersion is often required in studying spore ornamentation of *Russula* and *Lactarius* for example. When set up correctly the depth of focus of the X100 is so small that it is possible to focus on one surface of a spore at a time and thus not have the confusion of seeing the structures on both surfaces superimposed. With experience and confidence each person will have their own way of using the oil immersion lens but I will describe my own method.

After finding the appropriate field of study with the low power dry objectives lower the stage and carefully place a drop of oil on the slide (it sometimes helps to swing the X4 objective into position to give you more working space). Adjust the fine focus control to the highest position of its range. Swing the X100 into place and carefully raise the stage, watching from the side as you do so (Fig. 4). The bottom surface of the objective is flat, the surface of the drop of oil is curved and, as soon as they meet, surface tension causes the oil to spread rapidly over the lens. It almost looks as though the oil has jumped up to meet the lens! At this point look down the eyepiece and continue focusing down with the fine focus. Do not go too fast, the depth of field is very limited and, if you blink at the wrong time, you may go through the point of focus.

For completeness note that for the very best optical results the numerical aperture of the condensor and the objective should also be matched. When working with oil immersion, this would require oiling the gap between the condensor lens and the bottom of the slide, a messy business! I have to confess that I have not attempted this level of excellence. Although modern immersion oils are non-drying, it is essential to clean off the oil from the objective after use with lens tissues (do not be tempted to use ordinary tissue paper as this is not guaranteed to be free of fine particles of grit which may scratch the surface of the lens).

One final tip on using oil: it is more viscous than most mounting fluids so, as one

is focusing, there may be a tendency for the cover slip to be pushed down, thus moving those spores you were hoping to study! Use as little mountant and material as possible so that the cover slip is flat and as close to the slide as possible.

References

Bradbury, S. & Bracegirdle, B. (1998) *Introduction to Light Microscopy*. Bios Science Publishers, Oxford.

Moss, M.O. (2000) Setting up the microscope part 1. *Field Mycology* 1(4): 128-130.

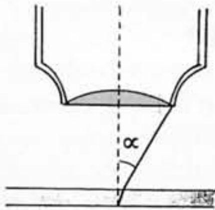


Fig. 1

The value of α in the Numerical Aperture of an objective

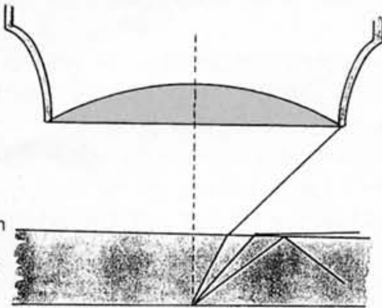


Fig. 2

Internal reflection of light passing from glass to air

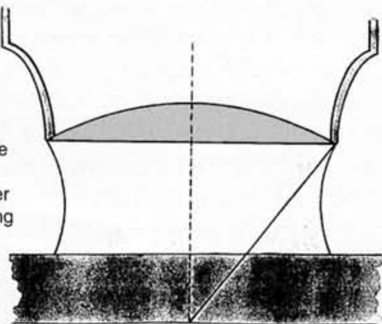
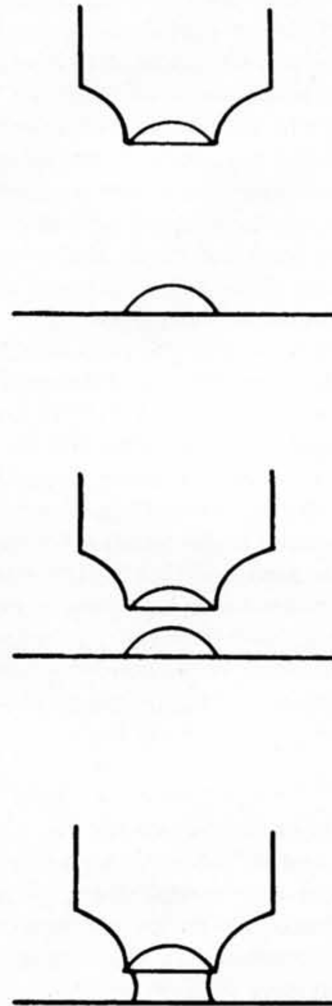


Fig. 3

Increasing the angle of the cone of light passing through the cover slip by introducing oil



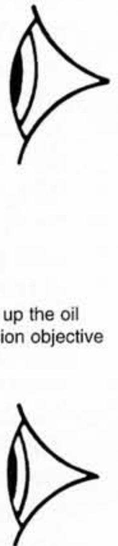


Fig. 4

Setting up the oil immersion objective