Chapter 2

Microscopy

2.1 INTRODUCTION

Visualization of microorganisms requires use of either the light microscope or the electron microscope. In general, light microscopes are used to look at intact cells at low magnification, while electron microscopes are used to look at internal structure or detail of the cell surfaces. All microscopes employ lenses to magnify the image of a cell so that details of its structure are more apparent. In addition to magnification, the ability to distinguish two adjacent objects as distinct and separate is important and known as resolution. Although magnification can be increased virtually without limit, resolution cannot; resolution is distinct by the physical properties of light. Thus, it is resolution and not magnification that ultimately dictates what we are able to see with a microscope. (Fig. 2.1)

2.2 STUDY OF LIGHT MICROSCOPE

Modern microscopes are all compound microscopes, i.e., the magnified image formed by the objective lens is further enlarged by one or more additional lenses. The light microscope, so-called because it employs visible light to detect small objects, is probably the most well-known and well-used research tool in biology. Since the cost of an instrument increases with its quality and versatility, the best instruments are, unfortunately, unavailable to most academic programmes. However, even the most inexpensive "student" microscopes can provide spectacular views of nature and can enable students to perform some reasonably sophisticated experiments.

A beginner tends to think that the challenge of viewing small objects lies in getting enough magnification. In fact, when it comes to looking at living things, the biggest challenges are, in order to obtaining sufficient contrast, finding the focal plane, obtaining good resolution and recognizing the subject when one sees it.

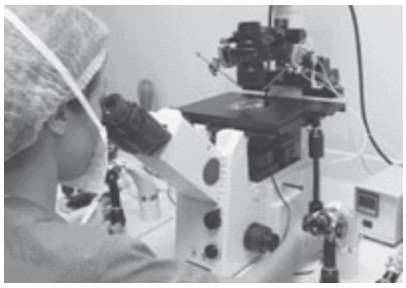

Fig. 2.1 Student working with microscope.

2.3.2 Using a Bright Field Microscope

First, think about what you want to do with the microscope. What is the maximum magnification you will need? Are you looking at a stained specimen? How much contrast/resolution do you require? Next, start setting up for viewing. (Fig. 2.2a, b)

Mounting of specimen

The cover slip must be up. High magnification objective lenses cannot focus through a thick glass slide; they must be brought close to the specimen, which is why cover slips are so thin. The stage may be equipped with simple clips (less expensive microscopes), or with some type of slide holder. The slide may require manual positioning, or there may be a mechanical stage (preferred) that allows precise positioning without touching the slide.

Optimize the lighting

A light source should have a wide dynamic range to provide high intensity illumination at high magnifications, and lower intensities so that the user can view comfortably at low magnifications. Better microscopes have a built-in illuminator, and the best microscopes have controls over light intensity and shape of the light beam. If your microscope requires an external light source, make sure that the light is aimed toward the middle of the condenser. Adjust illumination so that the field is bright without hurting the eyes.

Adjust the condenser

To adjust and align the microscope, try using these guidelines. If the condenser is focusable, position it with the lens as close to the opening in the stage as you can get it. If the condenser has selectable options, set it to bright field. Start with the aperture diaphragm stopped down (high contrast). You should see the light that comes up through the specimen change brightness as you move the aperture diaphragm lever.

Think about what you are looking for

It is a lot harder to find something when you have no expectations as to its appearance. How big is it? Will it be moving? Is it pigmented or stained, and if so, what is its colour? Where do you expect to find it on a slide? For example, students typically have a lot of trouble finding stained bacteria, because with the unaided eye and at low magnifications, the stuff looks like dirt. It helps to know that as smears dry down, they usually leave rings so that the edge of a smear usually has the densest concentration of cells.

Focus, locate, and centre the specimen

Start with the lowest magnification objective lens, to home in on the specimen and/or the part of the specimen you wish to examine. It is rather easy to find and focus on sections of tissues, especially if they are fixed and stained, as with most prepared slides. However, it can be very difficult to locate living, minute specimens such as bacteria or unpigmented protists. A suspension of yeast cells makes a good practice specimen for finding difficult objects.

Start with the specimen out of focus so that the stage and objective must be brought closer together. The first surface to come into focus as you bring stage and objective together is the top of the coverslip. With smears, a coverslip is frequently not used, so the first thing you see is

the smear itself. If you are having trouble, focus on the edge of the coverslip or an air bubble, or something that you can readily recognize. The top edge of the coverslip comes into focus first, and then the bottom, which should be in the same plane as your specimen. Once you have found the specimen, adjust contrast and intensity of illumination, and move the slide around until you have a good area for viewing.

Adjust eyepiece separation, focus

With a single ocular, there is nothing to do with the eyepiece except to keep it clean. With a binocular microscope (preferred), you need to adjust the eyepiece separation just like you do a pair of binoculars. Binocular vision is much more sensitive to light and detail than monocular vision. So if you have a binocular microscope, take advantage of it.

One or both of the eyepieces may be a telescoping eyepiece, i.e., you can focus it. Since very few people have eyes that are perfectly matched, most of us need to focus one eyepiece to match the other image. Look with the appropriate eye into the fixed eyepiece and focus with the microscope focus knob. Next, look into the adjustable eyepiece (with the other eye of course), and adjust the eyepiece, not the microscope.

Select an objective lens for viewing

The lowest power lens is usually 3.5 or 4x, and is used primarily for initially finding specimens. We sometimes call it the scanning lens for this reason. The most frequently used objective lens is the 10x lens, which gives a final magnification of 100x with a 10x ocular lens. For very small protists and for details in prepared slides, such as cell organelles or mitotic figures, you will need a higher magnification. Typical high magnification lenses are 40× and 100×. The latter magnification is used exclusively with oil in order to improve resolution.

Move up in magnification by steps. Each time you go to a higher power objective, re-focus and re-centre the specimen. Higher magnification lenses must be physically closer to the specimen itself, which poses the risk of jamming the objective into the specimen. Be very cautious when focusing. By the way, good quality sets of lenses are parfocal, i.e. when you switch magnifications, the specimen remains in focus or close to focused.

Bigger is not always better. All specimens have three dimensions, and unless a specimen is extremely thin, you will be unable to focus with a high magnification objective. The higher the magnification, the harder it is to "chase" a moving specimen.

(a)

(b)

Fig. 2.2 a. Student viewing light microscope; b. A light microscope.

cell structures. A phase-contrast microscope converts slight differences in refractive index and cell density into easily detected variations in light intensity, and is an excellent way to observe living cells.

The condenser of a phase-contrast microscope has an annular stop, an opaque disk with a thin transparent ring, which produces a hollow cone of light. As this cone passes through a cell, some light rays are bent due to variations in density and refractive index within the specimen and are retarded by about ¼ wavelengths. The deviated light is focused to form an image of the object. Undeviated light rays strike a phase ring in the phase plate, a special optical disc located in the objective, while the deviated rays miss the ring and pass through the rest of the plate. If the phase ring is constructed in such a way that the undeviated light passing through it is advanced by ¼ wavelength, the deviated and undeviated waves will be about ½ wavelength out of phase and will cancel each other when they come together to form an image. The background, formed by undeviated light, is bright, while the unstained object appears dark and contrast. This type of microscopy is called dark-phase-contrast microscopy. Colour filters are often used to improve the image.

Phase-contrast microscopy is especially useful for the detection of bacterial components such as endospores and inclusion bodies containing poly-β-hydroxybutyrate, polymetaphosphate, sulphur, or other substances. These are clearly visible because they have refractive indexes markedly different from that of water. Phase-contrast microscopes are also widely used in studying eukaryotic cells.

2.7 THE FLUORESCENCE MICROSCOPE

The microscopes thus far considered produce an image from light that passes through a specimen. An object can also be seen because it actually emits light, and this is the basis of fluorescence microscopy. When some molecules absorb radiant energy, they become excited and later release much of their trapped energy as light. Any light emitted by an excited molecule will have a longer wavelength than the radiation originally absorbed. Fluorescent light is emitted very quickly by the excited molecule as it gives up its trapped energy and returns to a more stable state.

The fluorescence microscope exposes a specimen to ultraviolet, violet, or blue light and forms an image of the object with the resulting fluorescent light. A mercury vapour arc lamp or other source produces an intense beam, and heat transfer is limited by a special infrared filter. The light passes through an exciter filter that transmits only the desired wavelength. A dark field condenser provides a black background against which the fluorescent objects glow. Usually, the specimens have been stained with dye molecules called fluorochromes that fluoresce brightly upon exposure to light of a specific wavelength, but some microorganisms are auto fluorescing. The microscope forms an image of the fluorochromes labelled microorganisms from the light emitted when they fluoresce. A barrier filter positioned after the objective lenses removes any remaining ultraviolet light, which could damage the viewer's eyes, or blue and violet light, which would reduce the contrast of the image.

2.8 ELECTRON MICROSCOPE

Electron microscopes use an electron beam as a source of illumination instead of visible light, and they use magnets instead of lenses to focus the beam. In transmission electron microscope

2.9 CARE AND MAINTENANCE OF THE MICROSCOPE

Everything on a good quality microscope is unbelievably expensive, so be careful. Hold a microscope firmly by the stand only. Never grab it by the eyepiece holder, for example. Hold the plug (not the cable) when unplugging the illuminator. Since bulbs are expensive and have a limited life, turn the illuminator off when you are done. Always make sure the stage and lenses are clean before putting away the microscope. Never use a paper towel, a Kim wipe, your shirt, or any material other than good quality lens tissue or a cotton swab (must be 100% natural cotton) to clean an optical surface. Be gentle! You may use an appropriate lens cleaner or distilled water to help remove dried material. Organic solvents may separate or damage the lens elements or coatings. Cover the instrument with a dust jacket when not in use. Focus smoothly; do not try to speed through the focusing process or force anything. For example, if you encounter increased resistance when focusing, then you have probably reached a limit and you are going in the wrong direction.