Light Microscopy

Light microscopy is based on the interaction of light and tissue components and can be used to study tissue features.

Upon completion of this lecture, the student should be able to:

1- Explain the basic principles of each type of light microscopes.
2- Explain the additional materials required for the examination with each microscope.
3- Choose the appropriate microscope type according to the specimen type and aim of the examination.

Bright-Field Microscopy

With the **bright-field microscope** (e.g. Compound Microscope), widely used by students of histology, stained preparations are examined by means of ordinary light that passes through the specimen. The microscope is composed of **mechanical** and **optical** parts (Figure 1–3). The optical components consist of three systems of lenses. The **condenser** collects and focuses light, producing a cone of light that illuminates the object to be observed. The **objective** lenses enlarge and project the illuminated image of the object in the direction of the eyepiece. The **eyepiece** or ocular lens further magnifies this image and projects it onto the viewer's retina, photographic film, or (to obtain a digital image) a detector such as a charge-coupled device (CCD) camera. The total magnification is obtained by multiplying the magnifying power of the objective and ocular lenses.

The critical factor in obtaining a detailed image with a light microscope is its **resolving power**, defined as the smallest distance between two particles at which they can be seen as separate objects. The maximal resolving power of the light microscope is approximately 0.2um; this power permits good images magnified 1000–1500 times.

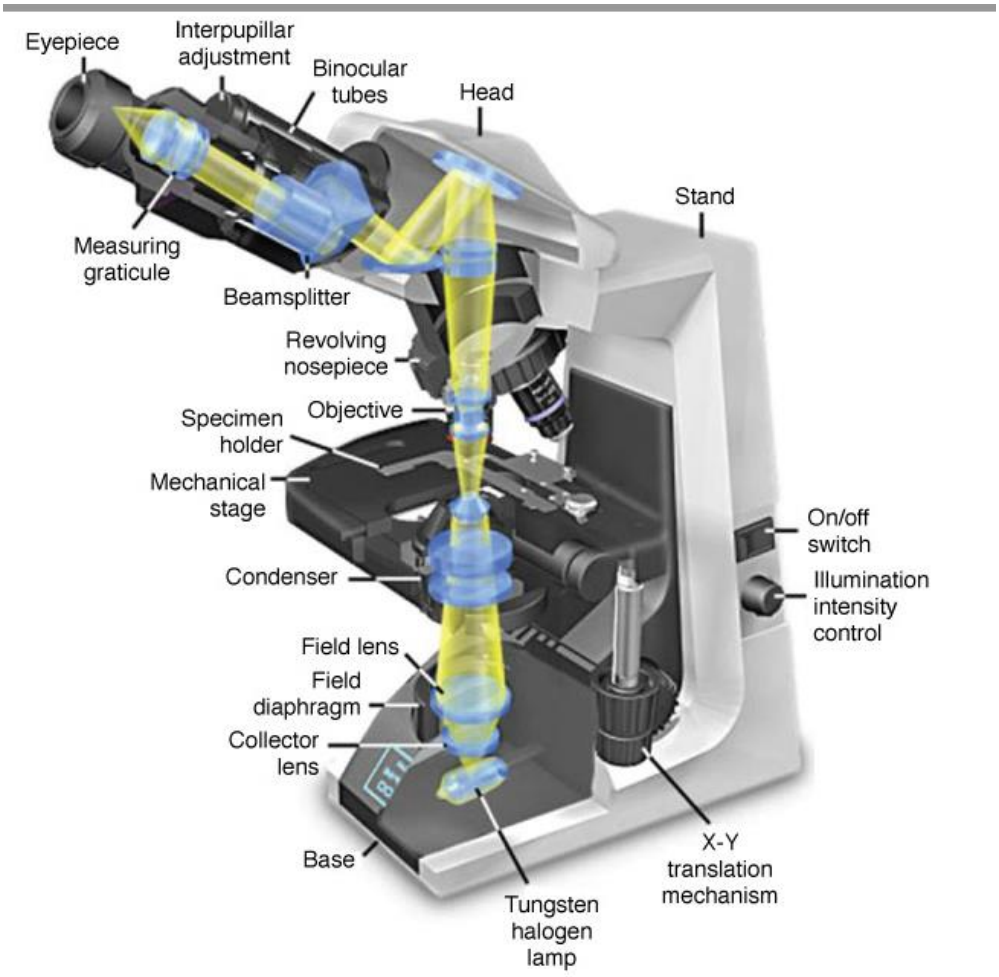

Figure 1-3: Bright field microscope

Objects smaller or thinner than 0.2um (such as a ribosome, a membrane, or a filament of actin) cannot be distinguished with this instrument. Likewise, two objects such as mitochondria will be seen as only one object if they are separated by less than 0.2 m. The **quality** of the image (its clarity and richness of detail) depends on the microscope's resolving power. *The magnification is of value only when accompanied by high resolution. The resolving power of a microscope depends mainly on the quality of its objective lens*. The eyepiece lens enlarges only the image obtained by the objective; it does not improve resolution. For this reason, when comparing objectives of different magnifications, *those that provide higher magnification also have higher resolving power*.

Video cameras highly sensitive to light enhance the power of the bright-field and other light microscopes and allow the capture of digitized images suitable for computerized image analysis and printing. The frontiers of light microscopy have been redefined by the use of such cameras. With digital cameras and image-enhancement programs (to enhance contrast, for example), objects that may not be visible when viewed directly through the ocular may be made visible in the video screen. *These video systems are also useful for studying living cells for long periods of time, because they use low-intensity light and thus avoid the cellular damage from heat that can result from intense illumination*. Moreover, software developed for image analysis *allows rapid measurements and quantitative study of microscopic structures*.

Fluorescence Microscopy

When certain substances are irradiated by light of a proper wavelength, they emit light with a longer wavelength. This phenomenon is called fluorescence. In **fluorescence microscopy**, tissue sections are usually irradiated with ultraviolet (UV) light and the emission is in the visible portion of the spectrum. *The fluorescent substances appear brilliant on a dark background.* For this method, the microscope has a strong UV light source and special filters that select rays of different wavelengths emitted by the substances.

Fluorescent compounds with affinity for specific cell macromolecules may be used as **fluorescent stains**. Acridine orange, which binds both DNA and RNA, is an example. When observed in the fluorescence microscope, these nucleic acids emit slightly different fluorescence, allowing them to be localized separately in cells (Figure 1–4a). Other compounds such as Hoechst stain and DAPI specifically bind

DNA and are used to stain cell nuclei, emitting a characteristic blue fluorescence under UV.

Another important application of fluorescence microscopy is achieved by coupling fluorescent compounds to molecules that will specifically bind to certain cellular components and thus allow the identification of these structures under the microscope (Figure 1–4b). Antibodies labeled with fluorescent compounds are extremely important in immunohistological staining.

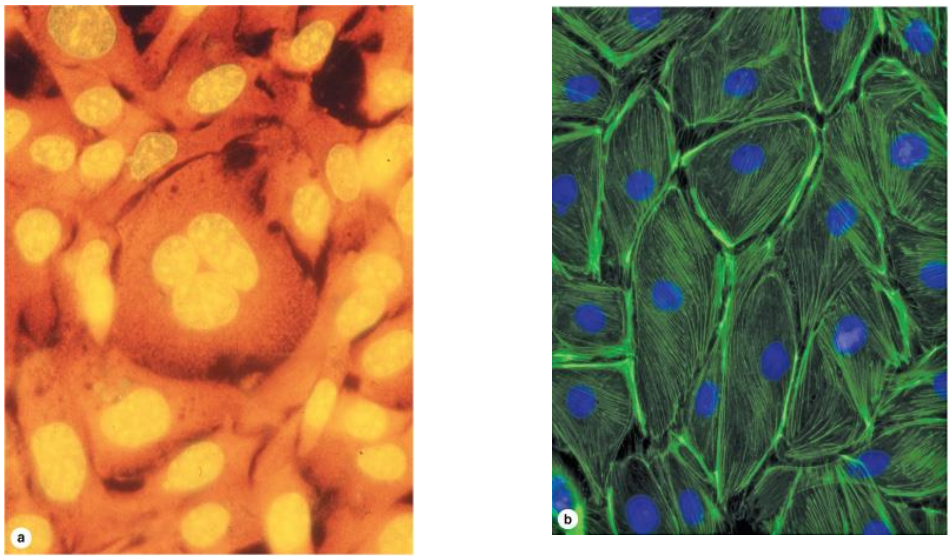

Figure 1–4: (a): Kidney cells stained with acridine orange. Nuclear DNA emits yellow light and the RNA-rich cytoplasm appears reddish or orange. (b): The less dense culture of kidney cells stained with DAPI. Nuclei of these cells show a blue fluorescence and actin filaments appear green.

Phase-Contrast Microscopy

Some optical arrangements allow the observation of unstained cells and tissue sections. Unstained biological specimens are usually transparent and difficult to view in detail, because all parts of the specimen have almost the same optical density. **Phase-contrast microscopy**, however, uses a lens system that produces visible images from transparent objects (Figure 1–5).

Phase-contrast microscopy is based on the principle that light changes its speed when passing through cellular and extracellular structures with different **refractive indices**. These changes are used by the phase-contrast system to cause the structures to appear lighter or darker in relation to each other. Because it does not require fixation or staining, phase-contrast microscopy allows observation of living cells and tissue cultures, and such microscopes are prominent tools in all cell culture labs.

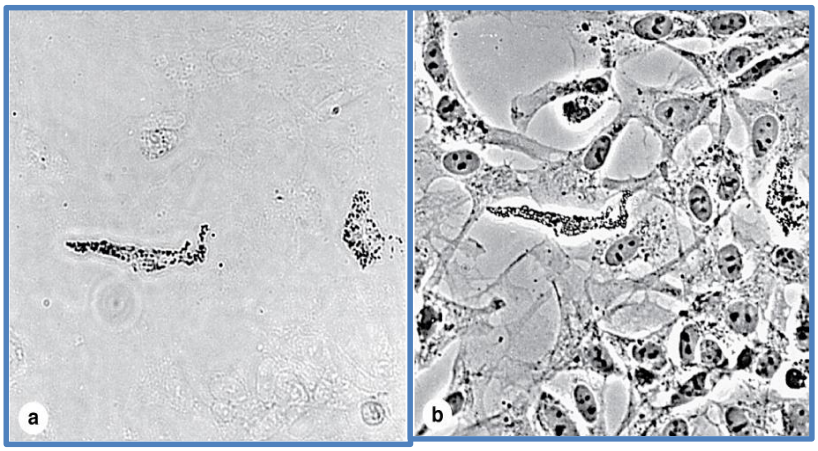

Figure 1–5: Cells under:- a- bright field microscope, b- Phase-contrast microscopy

Confocal Microscopy

With a regular bright-field microscope the beam of light is relatively large and fills the specimen. Stray light reduces contrast within the image

and compromises the resolving power of the objective lens. Confocal microscopy avoids stray light and achieves greater resolution by using: (1) A small point of high-intensity light provided by a laser and
(2) A plate with a pinhole aperture in front of the image detector. The point light source, the focal point of the lens, and the detector's pinpoint aperture are all optically conjugated or aligned to each other in the focal plane (confocal) and unfocused light does not pass through the pinhole. This greatly improves resolution of the object in focus and allows the localization of specimen components with much greater precision than with the bright-field microscope.

Most confocal microscopes include a computer-driven mirror system (the beam splitter) to move the point of illumination across the specimen automatically and rapidly. Digital images captured at many individual spots in a very thin plane-of-focus are used to produce an "optical section" of that plane. Moreover, creating optical sections at a series of focal planes through the specimen allows them to be digitally reconstructed into a three-dimensional image. Important features of confocal microscopes are shown in Figure 1–6.

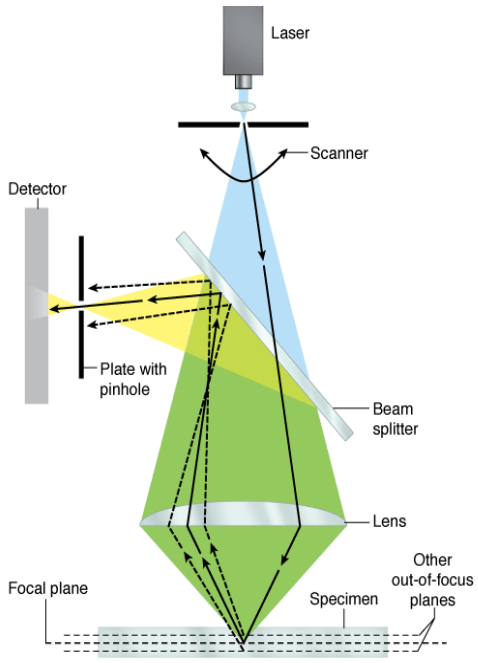

Figure 1–6: Confocal microscope