Question

1. Discuss the physics of the light microscope diagrams and illustrations needed.
2. Write notes on the ff biomedical equipment. Add notes on principle, brand, care and maintainance and cost
   a. Centrifuge
   b. Automatic Tissues processor
   c. Microtome

ANSWER

1. **Discuss the physics of the light microscope diagrams and illustrations needed.**

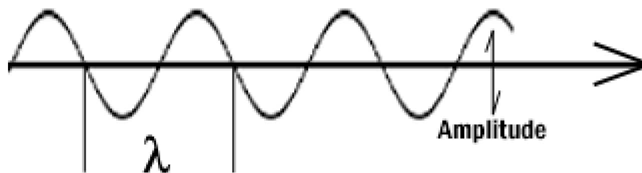
Light Microscopy

Introduction

Light microscopes play an important role in many research laboratories, including electron microscopy facilities. They can be used as a primary visualization tool or in support of electron microscopy. Samples for light microscopy are prepared in an ever-increasing number of techniques, and can range from sliced biological organisms and tissue cultures to materials science and geological samples. Light and electron microscopes share many similarities in their optical principles. Understanding how a light microscope works is not only critical for obtaining optimum light images, but also for understanding electron microscopy.

Principles of Light, Electrons, & Microscopy

In microscopy we take advantage of waveform properties of light. These waves when produced at a particular source vibrate at right angles to the line of propagation. Each wave has a peak and trough. The distance traveled forward by the light ray is one wavelength (lambda). Wavelength varies with the color and intensity of the source.



Schematic diagram of a wave.

How the image is formed

The structures the light microscope is called upon to resolve exert only a small influence on the light they transmit. What is changed is the phase of momentary vibration. Conventional brightfield illumination will lack contrast and the details of the sample remain invisible. When the emerging waves have acquired a larger phase difference due to changes in refractive index, greater contrast is produced. This manifests itself by an edge effect (diffraction, refraction, and reflection). Sample details may be resolved in a number of ways. When a light passes through stained structures intensity is reduced selectively depending on the color and density of the sample as the light is absorbed. Selective absorption of wavelengths of white light produces colored light. Refraction changes the direction of a light ray as it passes from one medium to

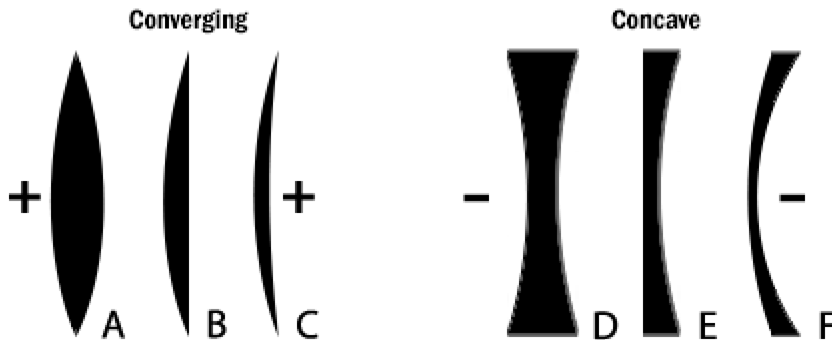
another. The shorter the wavelength, the greater the refractive angle. Diffraction is the bending of light rays around objects with sharp edges. A new wave front is created at this edge. Diffraction can be useful, but can also reduce resolution. When light is dispersed it is separated into its constituent wavelengths as a result of refraction on entering a transparent medium. Contrast can be defined as a steep slope between bright and dark image points. Adequate contrast MUST be achieved before the specimen can be resolved.
Two terms that are often confused, but are central to microscopy are magnification and resolution. Magnification is the degree by which dimensions in an image are, or appear to be, enlarged with respect to the corresponding dimensions in the object. Resolution is the act or result of displaying fine detail in an image. Magnification without resolution would be meaningless.
The theoretical resolution of the light microscope was first defined by Abbe in the following equation.

Abbe's equation for theoretical resolution of the light microscope:

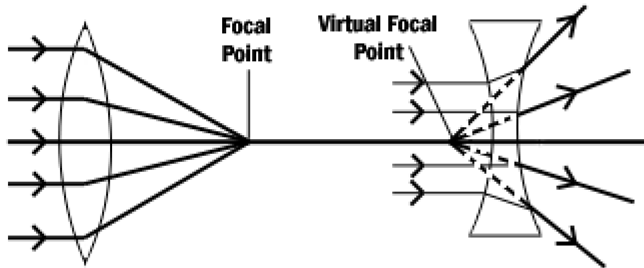
$$d = 1.22 \times \lambda o \, / \, n \sin \mu$$

d=distance of resolution
λ=wavelength of incident light
μ= 1/2 angle of incoming light

The actual resolution achievable with a light microscope is not as great. We will discuss the reasons for this later.
It is important to understand and to recognize the various components of the light microscope. The first and perhaps the most important element are the lenses.

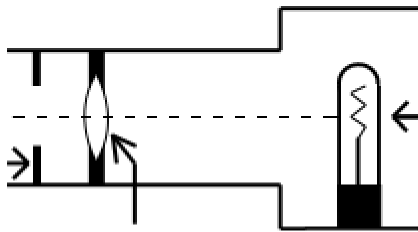


The six simple lenses. A, B, & C are converging or positive lenses. D, E, & F are concave or negative lenses.



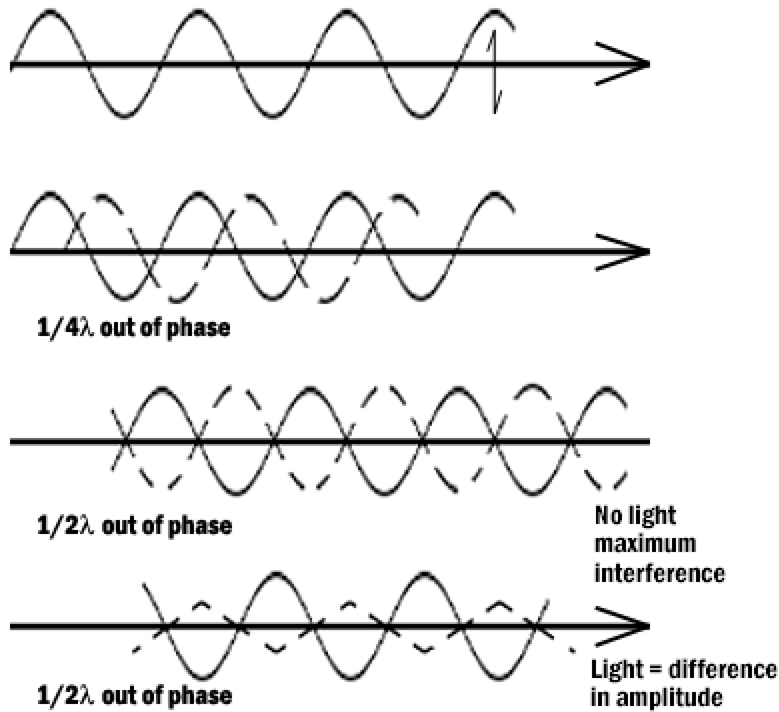
Converging and diverging lenses. Parallel rays enter a convex lens converge and meet at the principle focus. The distance between the center of the lens and the principal focus is the focal length. Parallel rays entering a concave lens diverge and never meet.

The Objective Lens is the first part of the imaging system; the objective lens forms a primary, enlarged image of the object. Very fine details are distinguished with the objective lens. The eyepiece sometimes called the ocular lens, is the second lens, which forms a secondary, further enlarged image. By multiplying the magnifying power of the objective lens and the magnifying power of the ocular the final magnification is found. A Substage Condenser lens is the third optical component. It is placed on a platform beneath the object. Light is directed through the substage condenser and converges to a point at the position of the specimen. The light rays diverge as they pass through the specimen and form an inverted cone, whose base is just large enough to fill the aperture of the objective. The size of the light beam is controlled by a diaphragm beneath the condenser called the aperture diaphragm.
The light source should contain both a lens to project an image of the lamp filament called a field condenser and a diaphragm to control the size of the illuminated field called a field diaphragm.



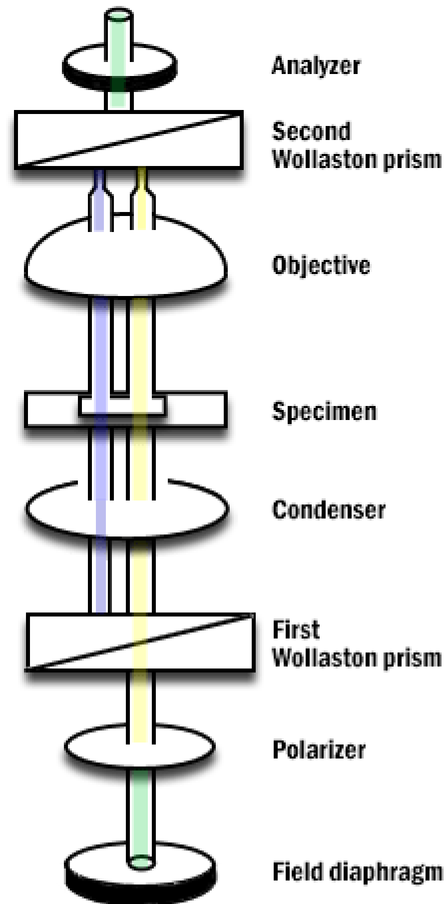
Typical lamp for light microscope.

Kohler Illumination is the most common method of illumination. In Kohler illumination the image of the source is projected by the field condenser onto the substage condenser, to the top of the plane of the object. This method assures even illumination.
Modern light microscopes use several different modes of operation depending on the needs of the investigator. The most common of these being brightfield microscopy in which direct light passes through the objective aperture and illuminates the background against which the image is seen. Since the structural elements being resolved have little variance in refractive index, the image will lack contrast and the details remain invisible. Small structure detail can be revealed by changing the absorption of the object by means of staining.
Many microscopes have special lenses that allow an investigator to image using phase-contrast. This method reveals details in specimens possessing very slight differences in optical path or refractive index in the surrounding media. Phase contrast requires no staining and can be used with living tissue. Contrast is produced in this method by phase differences in the light leaving the object to amplitude differences in the image. A phase plate alters the optical path length traveled by diffracted light from that traveled by direct light.



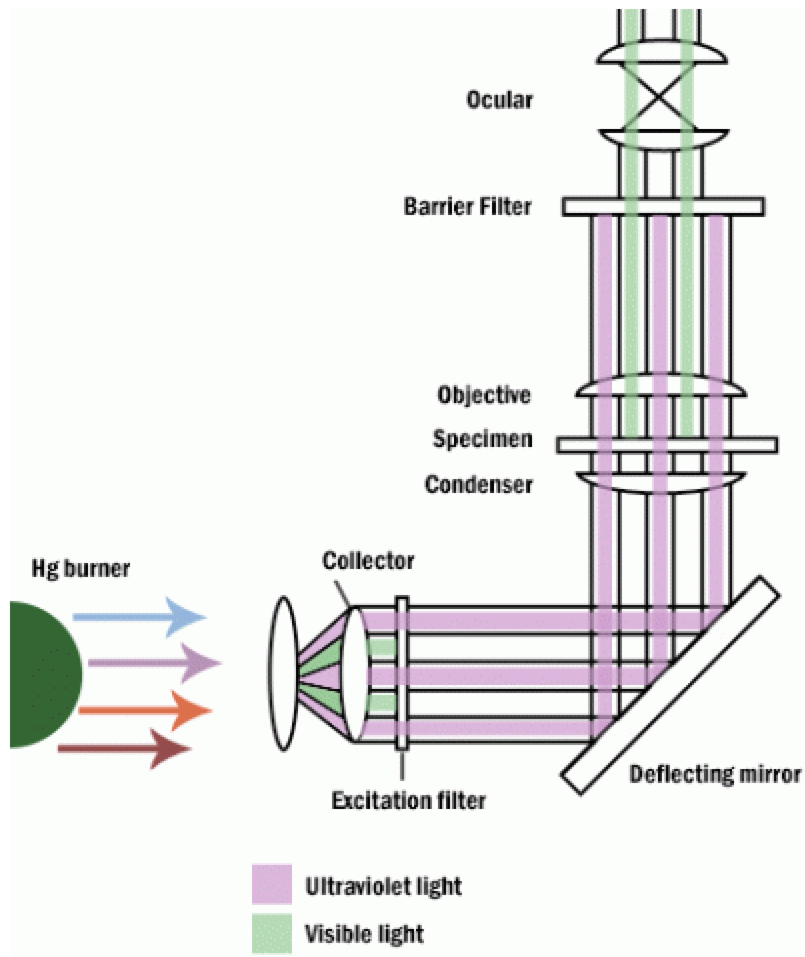
Light waves in brightfield and phase contrast after passing through an object. Pathway A represents the lightwave before encountering the object. Wave B represents the wave phase after passage in brightfield (unstained mode). C compares the wave phase of an object veiwed with phase contrast.

Differential interference contrast (DIC) differs from phase contrast in that the image has a strong relief and three-dimensional appearance. It must be remembered that the impression of surface details are the results of the optics and not the specimen for most biological samples. The optics for DIC consist of a polarizer at the light source and Wollaston prisms in the condenser and above the objectives. The beam passes through the polarizer, enters the first prism where it is split in two. One beam vibration is parallel to the prism and one is perpendicular. Both beams pass through the specimen in parallel in close proximity and are recombined in the second prism.

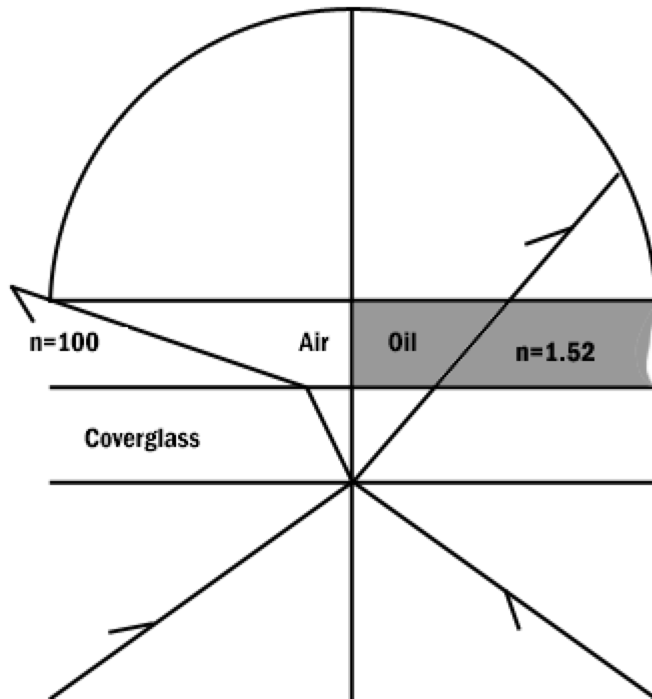


Differential Interference Contrast Schematic.

Darkfield microscopy is a mode in which direct light is prevented from passing through the objective aperture, but a hollow cone of light forms an apex in the plane of the specimen. The image is formed by light scattering from features of the object. Details appear bright white against a dark background. Darkfield can also be used for living bacteria, algae and plankton.
Some materials produce light when excited by short wavelengths of radiation. This effect is called fluorescence or auto-fluorescence. Specimens that do not fluoresce by themselves may be treated with fluorochromes which produce a secondary florescence. By illuminating with a high intensity mercury or xenon source and filtering out all but the desired excitation wave length to contact the specimen, the resulting longer (less energetic) wavelengths of emission from the specimen its self veiwed. Fluorescence microscopy can be used to enhance particular organelles, immunocytochemistry, in-situ hybridization, enzyme cytochemistry and elemental localization.



Fluorescence microscope.



Comparison of a dry and an oil immersion objective.

Abbe in order to ease in identification of lens quality devised an equation for numerical aperture. Numerical aperture numbers can assist in comparing angles of dry, water immersion, and oil immersion objectives. Note the similarity to Abbe’s equation for theoretical resolution. This number is found on all objective lenses.

N.A. = n sin u

n = refractive index of medium

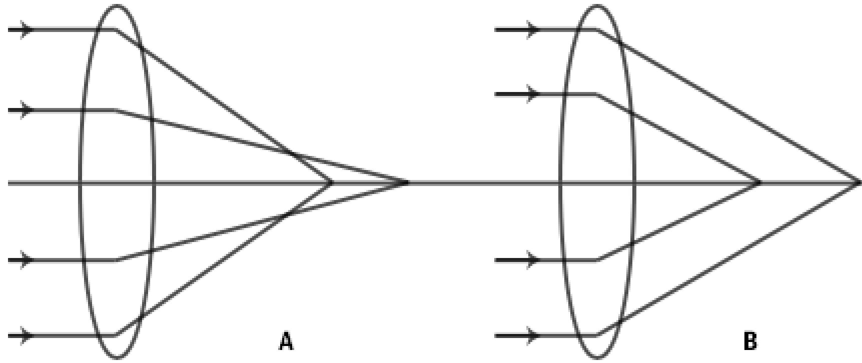
u = 1/2 the angle of light rays taken in when focused on the object.

When choosing an objective another consideration is depth of field. Depth of field is the distance from the nearest part of the subject in acceptable focus to the farthest part of the subject in acceptable focus. The efficiency (resolution) of a lens is inversely proportional to the depth of field (Table 1).

N.A.	**.25 .30 .50 .65 .85 .95**
Depth (in microns)	**8.0 5.5 2.0 1.0 .25 .10**

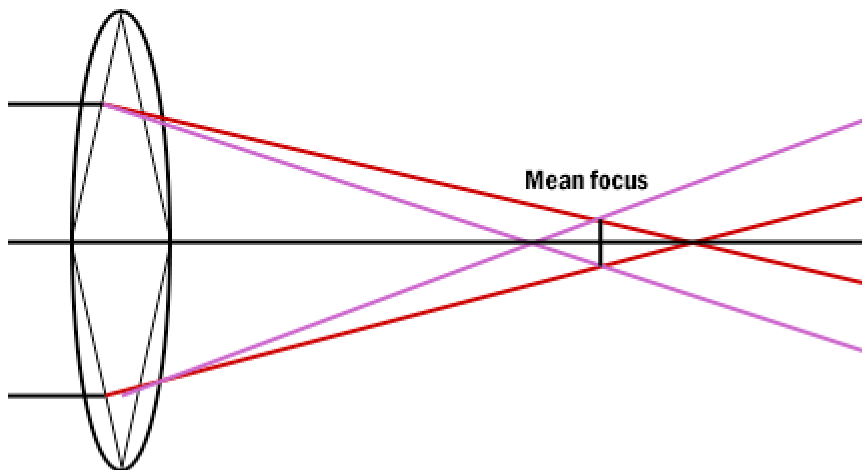
Variation in Depth of Field with Change in N.A.

Two aberrations within lenses detract from Abbe's equation of theoretical resolution. These aberrations are called spherical aberration and chromatic aberration. Spherical Aberration occurs when outer rays entering a lens are diffracted differently from those entering near the center. A solution for reducing spherical aberration is introducing a diaphragm or aperture.



Spherical aberration of a simple lens. A. Under correction. B. Over correction.

The thickness of the cover glass should be chosen according to specifications of a particular objective. Deviation from the required thickness results in over correction or under correction of spherical aberration. Chromatic Aberration occurs as white light entering a lens is broken into a spectrum from red to violet. Violet rays (more energetic) are refracted more than the red rays (less energetic). Consequently an uncorrected lens will be surrounded by color fringes. The more expensive lenses have a higher degree of correction.



Chromatic aberration of a simple lens. Each spectrum color has a separate focus.

2. Write notes on the ff biomedical equipment. Add notes on principle, brand, care and maintenance and cost.

- Centrifuge
- Automatic Tissues processor
- Microtome

Centrifuge:

PRINCIPLES OF CENTRIFUGATION

Particles suspended in a fluid move, under the influence of gravity, towards the bottom of a vessel at a rate that depends, in general, on their size and density. Centrifugation is a technique designed to utilise centrifugal forces, which are greater than the force of gravity, to speed up the sedimentation rate of particles. This is achieved by spinning the vessel containing the fluid and particles about an axis of rotation so that the particles experience a force acting away from the axis. The force is measured in multiples of the Earth's gravitational force and is known as the relative centrifugal field (RCF) or, more commonly, the '***g***' force.

Types of Centrifuge

Low-Speed Instruments

Low-speed centrifuges have maximum rotor speeds of less than 10,000 rpm, which do not require the rotors to be run in a vacuum, and there are instruments with a temperature control facility. Most instruments now include a sensor that will detect any imbalance when the rotor is running and cut ofifpower to the drive mechanism if imbalance is present. Low-speed instruments are used to separate serum or plasma from red blood cells, and to harvest and purify chemical precipitates, intact cells, nuclei, large mitochondria and large plasma-membrane fragments.

High-Speed Instruments

In general, high-speed centrifuges are capable of rotor speeds up to 21,000 rpm, although the new generation of super-speed instruments are capable of rotor speeds of 30,000 rpm, in which RCFs of 120,000 x***g*** are possible. These instruments require refrigeration systems to overcome the heat generated by the friction of the spinning rotor, and the higher-speed machines must incorporate vacuum systems. High-speed centrifuges are used in the separation of a number of cell constituents and in the isolation and purification of viruses.

Ultacentrifuges

Ultracentrifuges are capable of speeds in excess of 30,000 rpm and RCFs of over 600,000 x***g***.

They can be used in the isolation and purification of membrane components such as the endoplasmic reticulum and Golgi membrane, endosomes, ribosomes, DNA and RNA. Once again, refrigeration and vacuum systems are necessary.

PREVENTIVE MAINTENANCE

- If the bearings on the upper and lower ends of the motor shaft are not of the sealed type then they should be lubricated as per the manufacturer's instructions.

- Brushes should be removed regularly and checked for wear; they should be replaced if they are worn to more than one-half of their original length. When reinserting used brushes, replace them in the same orientation. New brushes should be broken in by slowly accelerating the unloaded unit to mid-speed and then allowing it to run for a period of time.
- The rotor, buckets and shields or carriers should be examined for signs of mechanical stress (eg cracks, corrosion).
- Some manufacturers etch the expiry date on the rotor and this should be checked periodically.
- Regularly lubricate the contact areas between the centrifuge buckets and Ihe pins.
- Regularly check the condition of the O-ring on the tie-down nut on top of the rotor, and replace it if worn or damaged.
- Always follow a manufacturer's specific instructions.

Care of Centrifuge

- **Avoiding Rotor Failures**
  The centrifugal field which accelerates the separation process also exerts large forces on the rotor material. If a rotor fails, the centrifuge is severely damaged as well. For this reason, some simple precautions should be observed
  Rotors are designed to be run up to their maximum speed with a load of a specific weight. One should never attempt to run a rotor at a speed higher than the one designated by its manufacturer. Also, if high density solutions (greater than 1.2 g/mL, for instance) are used, the run speed must be reduced to prevent undue stress on the rotor. Consult your instruction manual for exact directions.
- **Tube Breakage**
  Glass tubes can break during centrifugation, due either to improper loading or inherent defects. Any glass fragments must be removed from the buckets, adapters, rubber liners, and rotor chamber before the next run is made. If you find gray dust, which results from sandblasting of the rotor chamber by glass particles, it must be cleaned up too. You should make several dry runs without samples, and clean the chamber between each run to be sure this dust is eliminated from the centrifuge.
- **Chemical Resistance**
  If you plan to centrifuge any uncommon solvents or solutions, consult your manual to be sure they are compatible with the various plastics and metals comprising the centrifuge, the rotor, the tubes, and other accessories. These same precautions must be observed with any solvents used for sterilization purposes. A table of 19 chemical resistances for common centrifuge materials is available from Beckman Coulter.
- **Aerosol Generation**
  If any liquid is spilled on a rotor, it will be dispersed as a particulate mist when the centrifuge is run. Part of this mist will be fine enough to form a relatively stable aerosol which will tend to be dispersed throughout the laboratory. Such spills should be thoroughly cleaned up before running the centrifuge.
- **Handling Human Samples**
  Human blood or blood components can transmit an infectious disease or virus if the patient or donor carries these. Blood should be handled with respect for this possibility during all laboratory manipulations, including centrifugation.
- **When in doubt, refer to your instruction manual**
  From time to time, you'll have questions about the actual operation and maintenance of your centrifuge. The instruction manual provided with each instrument is designed to answer these questions. It should be read before making your first run, and kept handy for future reference.

AUTOMATIC TISSUE PROCESSOR

A tissue processor is a device that prepares tissue samples for sectioning and microscopic examination in the

diagnostic laboratory.

PRINCIPLE OF ATUMOTATIC TISSUE PROCESSOR

MODEL- TP 1050 Leica processor model
The **principle** of **tissue processing** is to remove the extractable water from **tissue** specimens and replace it with a medium that solidifies to allow sectioning. It consists of 3 stages which are dehydration, clearing and infiltrating
Most ATPMs are easy-to-program interface. The Leica processor model has ten 1.8L (60.9oz.) reagent beakers and two 1.8L (60.9oz.) wax baths.
The tissue basket oscillates up and down in each station at three-second intervals to ensure thorough and even mixing of the reagents and optimum tissue infiltration.Infiltration time is separately programmable for each station. Up to nine programs may be run with immediate or delayed starting times.When it's time for tissue to be transferred to the next beaker or jar, the cover of the machine is raised up, and the lifting mechanism carefully removes the tissue basket and gently transfers it to the next beaker.When the infiltration time for any particular station is exceeded, a warning message will display, indicating the station number and excess time. Controls are arranged by functionality with an LCD to indicate operational parameters. Reagent container lids have seals to minimize operator exposure to hazardous fumes.Tissue basket immediately immerses in a station in the event of power loss to protect samples from drying out. When power is restored, program will resume. In the event of long-term power failure, wax is liquified. Capacity of tissue basket is 80 cassettes.

Vacuum configurations hasten infiltration, allowing pressure to be applied to any station in either manual or automatic operation. Fume control configurations extract fumes with a fan and pass them through an internal carbon filter.

For added efficiency, these models feature a two-part containment shield surrounding the reagent container platform.

MAINTENANCE OF AUTOMATIC TISSUE PROCESSOR

- Have a preventive maintenance done once a year by a service engineer authorized by Leica.
- Once your warranty period expires, we recommend to purchase a Leica Service Contract. For details please contact your local Leica Service Organization.
- Mop up spilled reagents immediately.
- Clean the instrument on a daily basis.
- Once a month, lift the carousel cover to its upper end position, clean the carousel axle with a clean-

ing cloth and subsequently apply a thin coat of
machine oil.

- Never attempt any repairs on the instrument of paraffin stations on your own - by doing so you will loose any warranty claims!

MICROTOME

Microtome is an instrument with the help of which sections of tissues are cut and the process of cutting thin sections is known as Microtomy. The thickness of sections produced during microtomy may be between fractions of 50-100 nm, in ultramicrotomy, to several 100 microns. The common range is between 5-10m but both the maximum and minimum thickness is limited by the consistency of relation of the thickness of sections to the nature of tissues. These sections are stained using suitable staining techniques followed by observing them under the microscope.

TYPES OF MICROTOMES –

1.) Rotary microtome

The Rotary microtome is so-called because of a Rotary action of the handwheel responsible for the cutting moment. The block holder is mounted on a steel carriage, which makes up and down in groves this type of instrument is the most ideal for routine and research work it is excellent for cutting serial sections.

Parts of the rotary microtomes

- Block holder
- Knife clamp screw
- Knife clamps
- Block adjustment
- Thickness gauge
- The angle of tilt adjustment
- Operating handle

Here the feed mechanism is activated by turning a wheel on one side of the machine. The knife is fixed with its edge fixed upwards and the object is moved against the knife rising and falling vertically.

One rotation of the operating wheel produces a complete cycle downwards cutting stroke and an upward return stroke and activation of the advanced mechanism. It is often modified to cut ultrathin sections between

50Å – 200Å

The wheel may be electrically operated or manually. In the former case the hands may be made free for tissue maintenance, makes it available for incorporation in automated cryostats.

Advantages of the Rotary microtome

- Heavy and stable.
- Ideal for serial sections in large numbers.
- Paraffin-embedded tissues are cut by a rotary microtome.
- The knife holder is movable.
- The sections are cut are flat.
- It is useful for routine and research papers.

2.) Sliding or Base Sledge Microtome

This is a large heavy instrument with a fixed knife beneath which the object moves mounted on a heavy sliding base containing the feed mechanism used primarily for cutting the sections of cellulose nitrate embedded tissues with an obliquely set knife.

Parts of Base-sledge microtome

- Angular tilt adjustment
- Knife clamps
- Block holder
- Coarse feed adjustment
- Operating handle
- Thickness gauge
- Adjustment locking nut
- Block adjustment screw
- Split nut clasp

The blocks holder is mounted on a steel carriage which slides backward and forwards on groups against a fixed horizontal knife this microtome is heavy and very stable. The block is raised towards the knife at a predetermined thickness. This type of microtome is designed for cutting sections of very large blocks of tissues for example whole brain, this microtome has become popular for routine use.

Advantages of Base-sledge microtome

- It is useful for cutting extremely hard blocks and large sections.
- The microtome is heavy and stable.
- The knife used is sledge shaped which requires less honing.

3.) Cambridge rocking microtome

The instrument is so named because the arm has to move in a rocking motion while cutting the sections. The instrument was invented by Sir Horace Darwin in 1881 was developed by Cambridge company hence it is called the Cambridge rocking microtome. It is a simple machine in which the knife is held by means of microtome thread. The rocking microtome was designed primarily for cutting paraffin wax sections but in an emergency use frozen section by inserting a wooden block in which the tissue is frozen.

Parts of the rocking microtomes

- Knife holder
- Block holder or chuck
- Upper arm
- Screw
- Lever
- Pawl
- Ratchet wheel
- Mil head microtome screw
- Sleeve
- Lower Arm
- Scale

It cuts the sections between 1 to 20 microns. The knife is fixed with the edge, while the object is moved against this knife circularly, producing a sharply curved surface to the block with each stroke the tissue holder automatically moves vertically towards the life. Cutting stroke is Spring operated and is easy to handle. The microtome must be placed on a solid non-slippery surface to allow a better hold

Advantages of Cambridge rocking microtomes

- The cost of a knife and microtome is low.

- Celloidin embedded tissues can be sectioned easily.

4.) Freezing microtomes

This type has been designed for the production or preparation of frozen sections of fluid and non-fluid tissues usually without preliminary embedding. The object stage is connected to the cylinder of compressed carbon dioxide for the rapid cooling of the tissues and provisions are also made for the cooling of the knife.

Part of freezing type microtome

- Knife clamps
- Operating handle
- Thickness gauge
- Stage
- Stage valve
- Coarse adjustment

The movement of the knife takes place horizontally across the surface of the tissues. Ribbon sections cannot be prepared using this microtome. All freezing microtomes have the feature of employing a non-movable tissue block and cooling system.

Advantages of Freezing microtome

- It is used for sections required for Rapid diagnosis
- It cuts non-dehydrated fresh tissue in a frozen state.
- The method is useful for Rapid histopathological diagnosis during operation
- This type of microtome is also used when lipids, enzymes, and neurological structures are to be demonstrated.

Nowadays, the most commonly used type of microtome is a Rotary microtome which is easy to operate and ideal for routine use for diagnosis and research purposes.

BRAND- RM2125 RTS
CARE AND MAINTENANCE

- Clean all components daily – particularly the knife holder and specimen holder.
- Make sure that anything requiring locking down is locked down properly.
- Before using your knife, take a lint-free facial tissue saturated in either zylene, benzene or acetone to remove the protective oil coating on the knife. Use a dry, lint-free, facial tissue to wipe your knife clean. DO NOT USE GAUZE or any other coarse material; it will destroy the edge of your knife.

- Lock wheels and guard and remove the blade from the blade holder
- If applicable, the blade ejector can aid in removing the blade from the holder. Then, use
- forceps or a magnet (NEVER FINGERS) to pick up the blade.
- Blades must be stored in the appropriate blade case, or discarded into a sharps container.
- Remove the remaining tissue block.
- Clean the section waste tray with a dry brush.
- Use appropriate cleaning reagents; make sure no liquids enter the inside of the microtome.
- Reagents will be disposed as chemical waste, used blades will go into a sharps container and discarded tissues will be processed as biohazardous waste.
- Never attempt catching a falling blade.
- Make sure the surrounding area is clean and free of sharp objects.