Micro technique

(الثانية كيمياء وعلم الحيوان)

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(الجزء النظري)

الفصل الدراسي الاول

اعداد
د. مريم محمد جاد الرب

كلية العلوم

قسم علم الحيوان

2022-2023

بيانات الكتاب

الكلية: العلوم

الفرقة: الثانية

التخصص: كيمياء وعلم الحيوان

تاريخ النشر: الفصل الدراسي الاول

2023-2022م

عدد الصفحات:

الرموز المستخدمة

نص للقراءة والدراسة

اسئلة للتفكير والتقييم الذاتي

رابط خارجي

. Introduction

- Histology is the branch of anatomy that focuses on the study of tissues of animals and plants.
- The term tissue refers typically to a collection of cells. In humans, organs comprise two or more tissue types, including epithelial, connective tissue, nervous, and muscular.
- The word “histology” stems from the Greek word “Histo,” meaning web or tissue, and “logia,” meaning branch of learning. In brief, histological processing involves obtaining fresh tissue, preserving it (i.e., fixing it) in order to allow it to remain in as life-like a state as possible, cutting it into very thin sections (3–8 microns), mounting it on glass microscopic slides, and then staining the sections so that they can be observed under a microscope to identify different histological components within the tissue.

2. Techniques:

Preparing the tissue

- For tissue removal, it is necessary to gather first the informed consent of the patient, as tissue taken from a live individual for diagnosis or treatment requires his/her consent. In other words, the patient must know at the time he/she consents, the purpose of tissue removal.
- Similarly, harvesting tissue from an animal requires approval of the procedure by the institutional review board.
- An important first step in the histological process is tissue acquisition. This step can be achieved by means of traditional tissue dissection or endoscopic ultrasound (EUS)-guided fine needle aspiration.
- If the former dissection method is chosen, it is important to ensure that sharp dissecting tools are used to minimize crushing the tissue

while cutting for removal. The tissue should be kept moist (e.g., 0.85%saline, isotonic) while dissecting and trimming.

- The tissue should be trimmed 1–2 cm in width/length (but should not be more than 5 mm thick). There should be at least one to two cut sides for easy penetration of the fixative. It is important, at this stage, to determine the desired orientation of the tissue and that all tissue components are represented during this trimming stage, if possible.

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Fixation

- It is important to maintain cells in as life-like a state as possible and to prevent post-mortem changes as a result of putrefaction (destruction of tissue by bacteria or fungi) and autolysis (destruction of tissue by its own enzymes). In the latter case, as cells die, they release enzymes from their lysosomes and other intracellular organelles, which start to hydrolyze (i.e., break down or decompose by reacting with water) components of the tissue, such as proteins and nucleic acids with the help of proteases and nucleases, respectively.
- Cases of autolysis are most severe in tissues rich in enzymes (e.g., liver, brain, kidney, etc.) and are less rapid in tissues such as elastic fibers and collagen.
- Therefore, it is critical that fixation be carried out as soon as possible after removal of the tissues to prevent autolysis and putrefaction, as well as to prevent the tissue from undergoing osmotic shock, distortion, and shrinkage. Unfortunately, fixatives may, unintentionally, introduce artifacts which can interfere with interpretation of cellular ultrastructure.
- As fixation is typically the first step to prepare the tissue for microscopic or other, analysis, the choice of fixative and fixation protocol is very important.
- The fixative acts to denature proteins by

1- coagulation (of secondary and tertiary protein structures to form insoluble gels)
2- forming additive compounds (cross-linking end-groups of amino acids), or

3- A combination of coagulative and additive processes.

- In addition, fixatives promote the attachment of dyes to particular cell components by opening up protein side groups to which dyes may attach, remove bound water to increase tissue refractive index to improve optical differentiation, and alter the refractive index of tissues to improve contrast for viewing without staining.
- Prolonged fixation may result in the chemical masking of specific protein targets and prevention of antibody binding during immunohistochemistry protocols.
- In such cases, alternative fixation methods may be incorporated depending on the biological material. Therefore, there is no universal fixative which will serve all requirements. Each fixative has specific properties and disadvantages.
- There is no single fixative, or combination of fixatives, that has/have the ability to preserve and allow the demonstration of every tissue component. Some fixatives have only special and limited applications, while mixtures of two or more reagents may be necessary to employ the special properties of each. So, it is important to identify specifically which histological structures one is trying to demonstrate, as well as the effects of short-term and long-term storage of the tissues.
- When tissue is fixed, it is important to keep the sample size small, if possible, as increased thickness will retard fixative penetration. The volume of the fixative should be 20–25 times the volume of the tissue.
- The peritoneum or capsule around the tissue should be removed or pierced.
- The blood and mucus should be rinsed off with saline. The tissues should be cut with a new, sharp razor blade/scalpel, rather than scissors, as the latter could result in squeezing of the tissue, causing damage.
- Some tissues/organs will require special handling to ensure that the fixative reaches all internal components. Care should be given to ensure that the specimen has one or more cut sides to guarantee good penetration of the fixative.

- Sometimes, an agitating instrument can be employed to ensure that the fixative reaches all surfaces. At no time should the tissue be allowed to dry out.
- Each fixative will have its own fixation time and post fixation treatment for best preservation of cellular detail.
- Typical fixatives, depending on the type of tissue and microscopy technique intended, may include, formalin, Zenker's fixative, Bouin's fixative, Helly's fixative, Carnoy's fixative, Glutaraldehyde, osmium tetroxide, chromic acid, potassium dichromate, acetic acid, alcohols (ethanol, methanol), mercuric chloride, and acetone.

. Microwave irradiation:

- Microwave fixation has been found to be useful in increasing the molecular kinetics giving rise to accelerated chemical reactions.
- While conventional formalin-fixed, paraffin-embedded tissue offers superior cellular morphology and long-term storage, microwave-assisted tissue fixation with phosphate- buffered saline of normal saline offers the removal of the use of noxious and potentially toxic formalin fixation and a decrease in the turnaround time.
- In addition, staining of the microwave-fixed tissues was found to be sharper and brighter in most of the tissues than those obtained after conventional fixation [12, 14]. Interestingly, cold microwave irradiation procedures can offer rapid fixation and staining of tissues for electron microscopy and ultra-structural analysis.

Classification of fixatives

- Fixatives can be classified on the basis of three main criteria:
  - Action on proteins, types of fixative solution and, use.

1- Action on proteins

- Fixatives can have two main actions on proteins. They can be coagulant or non-coagulant
- Fixatives. Coagulant fixatives affect proteins in such a way that a coagulum (clot) forms.

- In contrast, non-coagulant fixatives result in a smoother “gel” formation.
- Cytoplasm is converted typically into an insoluble gel.
- In addition, while organelles are preserved, there is typically poor tissue penetration and artifacts are more likely to occur.

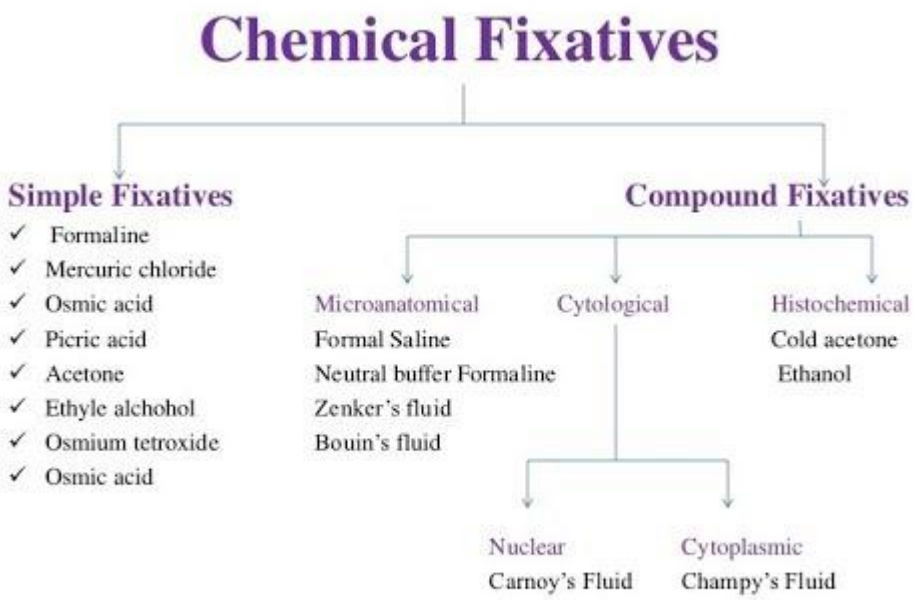
2- Types of fixative solution

- There are two main types of fixatives:
  - Primary and compound. Primary fixatives consist of a single fixative in solution.
  - Compound fixatives consist of two or more fixatives in solution, such as Zenker’s, Helly’s, and Bouin’s fixatives

3- Their use and mechanism of action

- The intention of micro anatomical fixatives is to preserve components of organs, tissues, or cells in spatial relation to each other.
- These fixatives are largely coagulant in nature (cell organelles are destroyed, typically), and used for light microscopy.
- Cytological fixatives, on the other hand, preserve cellular structures or inclusions, often at the expense of even penetration and allow the tissues to be cut relatively easily.
- They are non-coagulant in nature and are used typically for electron microscopy.
- They can be further subdivided into nuclear and cytoplasmic.

Types of fixatives



1) Aldehydes include formaldehyde (formalin, when in its liquid form), paraformaldehyde, and Glutaraldehyde. Tissues are fixed through cross-linking agents that react with proteins and nucleic acids in the cell (particularly lysine residues). Formaldehyde is a good choice for immune histochemical studies, while formalin is standard. The buffer prevents acidity in the tissues. Formaldehyde offers low levels of shrinkage and good preservation of cellular detail. This fixative is used routinely for surgical pathology and autopsy tissues requiring hematoxylin and eosin (H and E) staining, since formalin is toxic, carcinogenic, and a poor preserver of nucleic acids, there have been attempts to find a more suitable substitute; however, this has proved difficult.
2) Glutaraldehyde causes deformation of the alpha-helix structure in proteins, so it should not be used for immunohistochemistry staining. While it fixes very quickly, which makes it an excellent choice for electron microscopic studies, it provides poor penetration. It gives very good overall cytoplasmic and nuclear detail and is prepared as a buffered solution. This fixative works best when it is cold and buffered and not more than 3 months old.
3) Oxidizing agents include permanganate fixatives, such as potassium permanganate, dichromate fixatives (potassium dichromate), osmium tetroxide, and chromic acid. While these fixatives cross-link proteins, they cause extensive denaturation.

4) Alcohols, including methanol and ethanol, and protein denaturants (acetic acid) are not used routinely as they cause brittleness and hardness to tissues. They are useful for cytological smears, as they act quickly and provide good nuclear detail. Alcohols are used primarily for cytological smears. They are fast acting, cheap, and preserve cells through a process of dehydration and precipitation of proteins. Methanol has been shown to be effective during immune staining.

5) Mercurial fix tissues by an unknown mechanism. They contain mercuric chloride which is a known component in fixatives such as B-5 and Zenker's. These fixatives offer poor penetration and tissue hardness, but are fast and provide excellent nuclear detail, such as for visualization of hematopoietic and reticuloendothelial tissues. These fixatives must be disposed of carefully. Mercury deposits must be removed (degenderized) prior to staining; otherwise black deposits will occur in tissue sections.

6) Picrates include fixatives with picric acid, such as Bouin's solution. These fixatives have unknown modes of action. The most common is Bouin's alcoholic fixative. This fixative provides good nuclear detail and does not cause much hardness. It is recommended for fixation of testis, gastrointestinal tract, and endocrine tissues. This fixative has an explosion hazard in dry form, so it must be kept submerged in alcohol at all times.

7) Other factors affecting fixation

a- Buffering: Fixation is best performed at close to neutral pH (pH 6–8; formalin is buffered with phosphate at pH 7). Common buffers include: phosphate, bicarbonate, cacodyl ate, and vernal.

b- Penetration: Each fixative has its own penetration rate in tissues. While formalin and alcohol penetration are superior, Glutaraldehyde is the worst. Mercurial fixatives are in between. The thinner the sections are cut, the better the penetration.

c- Volume: The volume of the fixative should be in at least a ratio of 10:1. Fixation can be enhanced if the fixative solution is changed at regular intervals and the specimen is agitated.

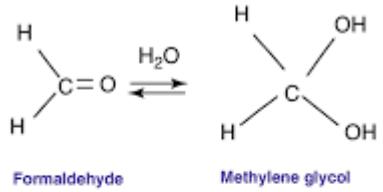
d- Temperature: If the temperature at which fixation is carried out is increased, it will yield an increased speed of fixation. Of course, too much heating of the fixative can result in cooking or creating tissue artifacts.

e- Concentration: The concentration of the fixative should be as low as possible, because too high a concentration may adversely affect the tissue and provide artifacts.

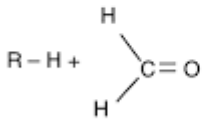
f- Time interval: The faster the fresh tissue can be acquired and fixed, the better, as to minimize cellular organelle degradation and nuclear shrinkage, resulting in artifacts. The tissue should always be kept moist with saline.

Decalcifying agents

- Some animal tissues contain deposits of calcium salts which may interfere with sectioning, resulting in torn sections and damaged blades. Calcium compounds must be chemically removed (usually with an acid) before typical histological techniques can be used for the study of softer components.
- Tissues requiring decalcification include bone, teeth, and calcified cartilage.
- Pathological states include arteriosclerosis, tuberculosis, and several tumor types. Such tissues should be fixed prior to decalcification and washed for 12 hours in running water between fixation and decalcification. While decalcification agents remove typically calcium salts and do not interfere with staining reactions, they can cause minimal distortion to cells and connective tissue. The decalcifying agent should have a volume of 30–50 times that of the tissue and occasional agitation may be required to expedite this process.
- Heating should not be employed. The process is complete typically when bubbling has ceased. Over decalcification can cause a severe reduction (of what) in subsequent sectioning of the tissue.
- Some typical decalcifying agents include nitric acid, Gooding and Stewart's fluid, Rapid Bone Decalcified (RDO), and chelating agents. More recently, new methods have been discovered to allow hard tissues to be decalcified faster.



Formaldehyde Methylene glycol



$= R - CH_2OH$

Bound hydroxymethy group

$R - CH_2OH + R - H = R - CH_2 - R + H_2O$

Metyhylene bridge

Dehydration

3- Dehydration.

❖The aim of dehydration is to replace all water in the tissue block with alcohol.

❖In ascending grades of alcohol (50% → 70% → 90% → 100%

❖Minimize shrinkage and distortion of tissues.

- After fixation, and to begin the dehydration step (i.e., removal of water), tissues are placed in progressively increasing concentrations of a dehydrating agent which is typically ethanol. Methanol, isopropanol, and acetone are alternative options, depending on the tissue being processed. It is important to include two absolute alcohol steps to ensure that all remaining water has been removed.
- The dehydration step is critical, as water is immiscible with most embedding media (i.e., paraffin wax).
- Therefore, the tissue must be exchanged between polar (e.g., water) and non-polar agents.
- If the tissue is incompletely dehydrated, it is not possible to “clear” the tissue. When it is exposed to a subsequent clearing agent the tissue remains opaque and appears milky. This will necessitate re-dehydration of the tissue.
- Dehydration will also remove some of the lipoid material in the tissue.

- If the lipids are supposed to be visible, it will be necessary to use an appropriate fixative that will preserve the lipids prior to the dehydration step.

. Clearing

- The term “clearing” is related to the appearance of the tissue after it has been treated with a dehydrating agent.
- Many agents have a similar refractive index to that of the tissue, rendering the tissue “clear” or translucent.
- In this step, the dehydrating agent must be removed from the tissue and replaced with a solvent of wax.
- A clearing agent should be used when the dehydrating agent is not miscible with the impregnating medium/ embedding agent.
- It is a wax solvent and must be miscible with both the dehydrating and embedding agents.
- The selection of a suitable clearing agent should be based on the speed and ease of removal from the embedding media, interaction with the tissue, flammability, toxicity, and cost.
- The clearing step can be more effective with the use of a vacuum system and should be carried out in a fume hood.
- Typical clearing agents include xylene, chloroform, toluene, benzene, dioxane, carbon tetrachloride, cedar wood oil, isoamyl acetate, methyl benzoate, methyl salicylate, and clove oil.
- Due to the potential hazards of some of these chemicals, others have been proposed, such as some vegetable oils, terpenes, and alkanes.
- Some histological protocols have the potential option of processing the tissue without the use of a clearing agent. As a safe alternative to exposure to the hazardous effects of these chemicals.
- One such protocol includes the use of isopropanol as a safer alternative

Infiltration/impregnation

- The role of the infiltration agent is to remove the clearing agent from the tissue and to completely permeate the tissue with paraffin wax.
- This will allow the tissue to harden and produce a wax block from which thin histological sections can be cut.

- Ideally, the consistency of any solidified embedding medium should be the same as the specimen it encloses.
- Unfortunately, this rarely happens due to the wide variation in consistency of tissue and the large variety in embedding media.
- Paraffin wax is commonly used and heated to a temperature that is 2–3°C above its melting point. Any higher temperature will result in tissue hardening.
- The paraffin wax should be 20–25 times the volume of the tissue.
- Generally, the tissues are transferred directly from the clearing agent to pure paraffin, but sometimes with fragile specimens, it is necessary to use graded mixtures of clearing agent and paraffin.
- The duration and number of changes of paraffin necessary for impregnation vary with the size and consistency of the tissue.
- As exposure of the tissue to paraffin increases, it is more likely that shrinkage and hardening will occur.
- Complete infiltration is only possible after complete dehydration and complete clearing.
- The selection of paraffin depends on the nature of the tissue to be embedded and thickness of section required.
- A high melting point of the wax increases the hardness and decreases the thickness to which the tissue may be sectioned.
- Paraffin wax can be purchased in the form of tablets, pellets, or granules.
- Numerous substances can be added to the molten paraffin to modify its consistency and melting point.
- Typically, the process of infiltration occurs with the use of a tissue processing machine, although this can be carried out using a heated container maintained 2–3°C above the melting point of wax.
- If residual clearing agents remain in tissue or improper processing of the tissue has occurred, this will lead to difficulties with sectioning.
- Evaporation of the clearing agent, infiltration with paraffin wax, and removal of any air bubbles trapped in the specimen will be more completely alleviated if clearing and infiltration procedures are carried out at reduced pressure (under vacuum).

Block now ready for sectioning (microtomy)

Embedding

- After the infiltration process has been completed, it is necessary to obtain a solid block containing the tissue.
- To accomplish this, it is necessary to first coat a stainless steel histological base mold of suitable size to fit the tissue with glycerol or “mold release” to prevent adherence of the wax block containing the tissue to the metal mold upon solidification.
- Pre-warming of the metal block is advised to prevent premature solidification of the wax block.
- In addition, using warmed forceps to help press the tissue against the base of the metal mold, in addition to reducing the chance of premature solidification, helps with this process.
- Prior to beginning the infiltration process, an embedding cassette should be placed on top of the mold and labeled with the name of the tissue, fixative, and date.
- If an embedding unit (machine) is being used, the combined unit should be dispensed two-thirds full with molten paraffin.
- The specimen should be oriented in the metal mold to ensure that the tissue will be cut in the correct plane of section.

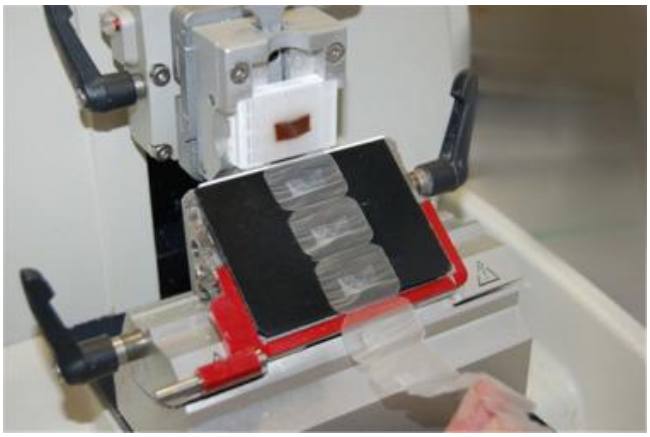
- Alternatively, the mold can be filled slightly and the tissue can then be placed in the mold and positioned in the desired orientation at the base of the mold.
- The combined unit should then be set out on the cooling tray of the embedding unit (machine) and not disturbed until the wax has cooled and solidified completely.
- After sufficient time, the cassette and mold should be separated and the paraffin block should be placed in the microtome in preparation for sectioning.
- If the tissue has been thoroughly fixed, dehydrated, cleared, and infiltrated, tissues embedded in paraffin wax provide good cutting qualities.
- On average, paraffin blocks remain durable and retain their good cutting qualities and staining characteristics indefinitely.

Embedding media

- The most common infiltrating agent and embedding medium is paraffin wax.
- Ester wax offers a lower melting point than paraffin wax and tends to be harder when solid, allowing this medium to be suitable for cutting thinner sections with minimal tissue shrinkage.
- When water-soluble waxes are used, tissues are transferred directly from aqueous fixatives to wax for infiltration without dehydration or clearing.
- This results in less tissue shrinkage, but sectioning is more difficult than with paraffin wax.
- Tissue blocks must be kept in a dry atmosphere. If cellulose nitrate is chosen as an embedding medium, tissues must be dehydrated and embedded with solutions of cellulose nitrate dissolved in an alcohol/ether mixture.
- The solvent is allowed to evaporate to produce a tissue block of required consistency. No heat is applied using this method.
- This medium is used typically for large pieces of, for example, bone and brain tissues.
- Synthetic resins are used for preparing sections most typically for electron microscopy and light microscopy, such as for UN decalcified bone.

- Freeze drying protocols can be applied when special staining techniques are used.

Microtome:

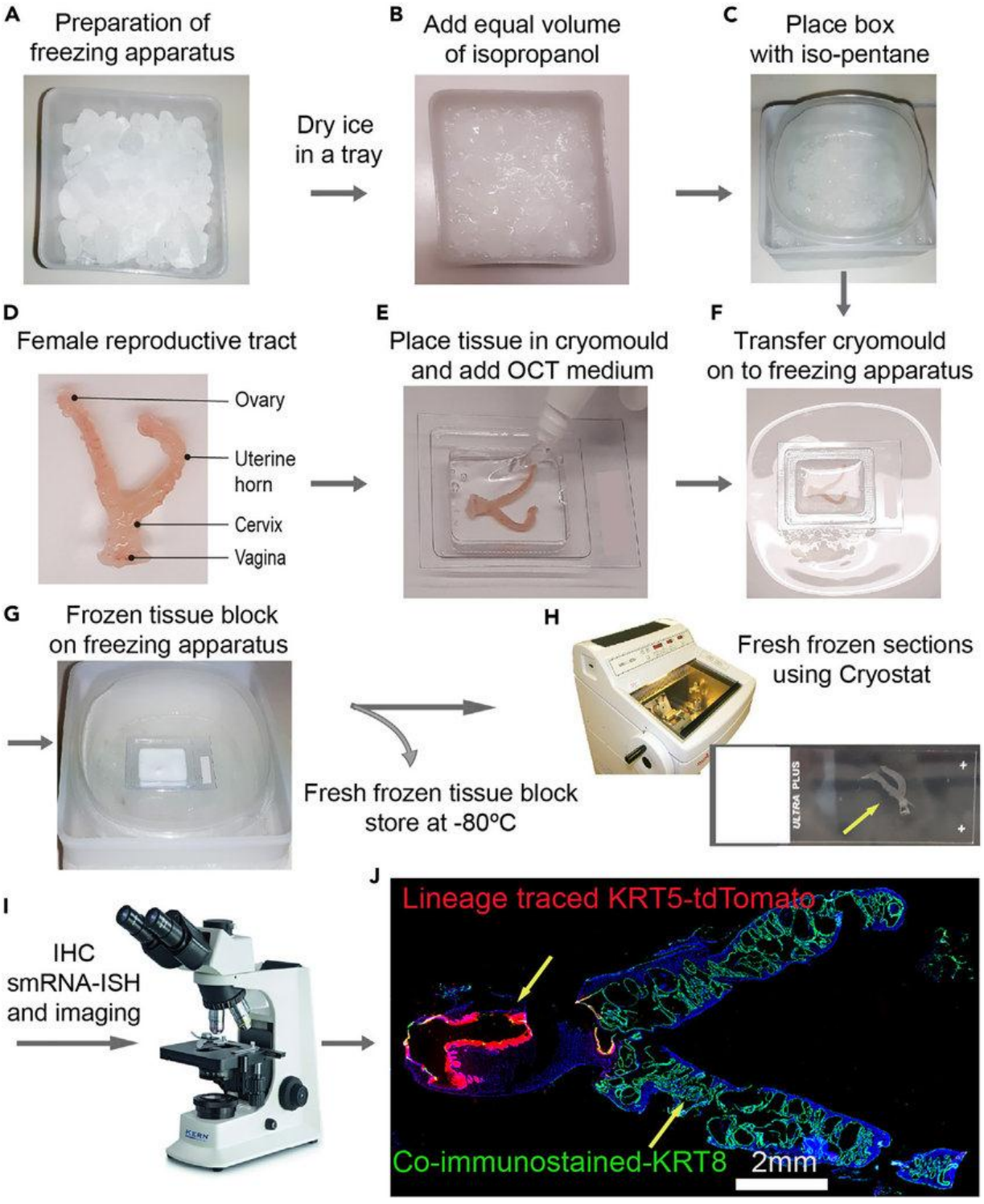


- Microtomes are used to cut the tissue into thin sections for microscopic viewing.
- The type of specimen will determine the type of microtome to be used. Rotary microtomes are the most common microtome instrument.
- The feed mechanism is achieved by turning a wheel at one side of the machine. While the knife is fixed and is secured in a knife holder, the object moves against the cutting surface of the knife, according to the thickness of section required.
- The knife holder allows the knife to be set at an oblique angle to the specimen. One complete rotation of the operating wheel is equivalent to one complete cycle.
- The downward motion of the knife reflects the cutting stroke, while the upward stroke reflects the return stroke and activation of the advance mechanism.
- The feed mechanism is activated by turning a wheel located on the side or top of the microtome.
- The tissue block is passed across the knife at every stroke to produce a section.

- Microtomes have a feed mechanism to advance the specimen (or knife) to a predetermined thickness for sectioning and can produce serial sections.

Cryostat

- A cryostat or freezing microtome is used for obtaining thin sections of unfixed tissues. It can be used, additionally, for observing fatty tissues.
- The microtome is maintained at −15 to−20°C in a refrigerated chamber. The cabinet is designed to operate at −5 to −30°C. The tissue block can be mounted in a high-viscosity water-soluble gel, such as 1% glucose, gelatin, or cellulose on the platform and must be frozen immediately.
- An anti-roll plate is used to keep sections flat on the knife blade for direct mounting onto the slide.
- Sections are cut one at a time. When a section is cut, the anti-roll plate is lifted and a section is picked up from the surface of the knife and placed onto a slide using a camel hair brush. Sections are fixed in 5% acetic acid in absolute alcohol and then subsequently stained.
- Frozen sectioning is typically used for rapid preparation and diagnosis by a pathologist.

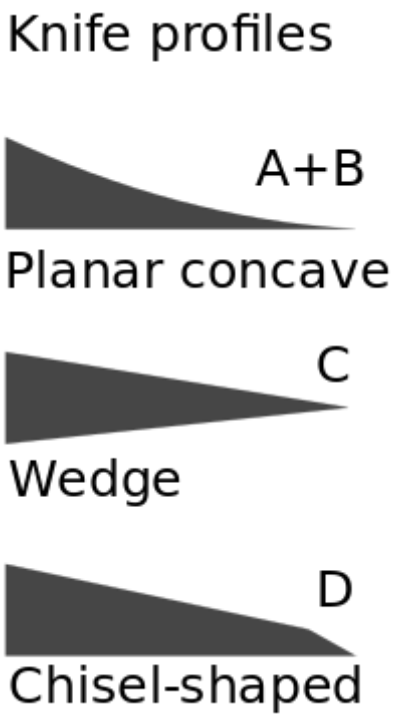


Microtome knives

- There are many different types of microtome knives (e.g., stainless steel, carbide, diamond, glass, or disposable blades). Wedge-shaped stainless steel knives are used for most paraffin embedded specimens. They must be kept clean and well-oiled or lubricated.
- The knife's edge should be cleaned with a clearing agent with a soft, moistened cloth in a fume hood. As an alternative to wedge-

shaped stainless steel knives, disposable blades provide an excellent cutting edge for paraffin sectioning and are available in different sizes and thicknesses.

- Glass, sapphire, and diamond knives are used for specimens embedded in hard resin plastic. Diamond and sapphire knives tend to function better than glass knives, but are much more expensive.
- If a wedge-shaped stainless steel knife is used, it must be free of nicks and sharpened with a carborundum stone (manual sharpening) or by an automatic knife sharpener (with a glass wheel and with an abrasive).

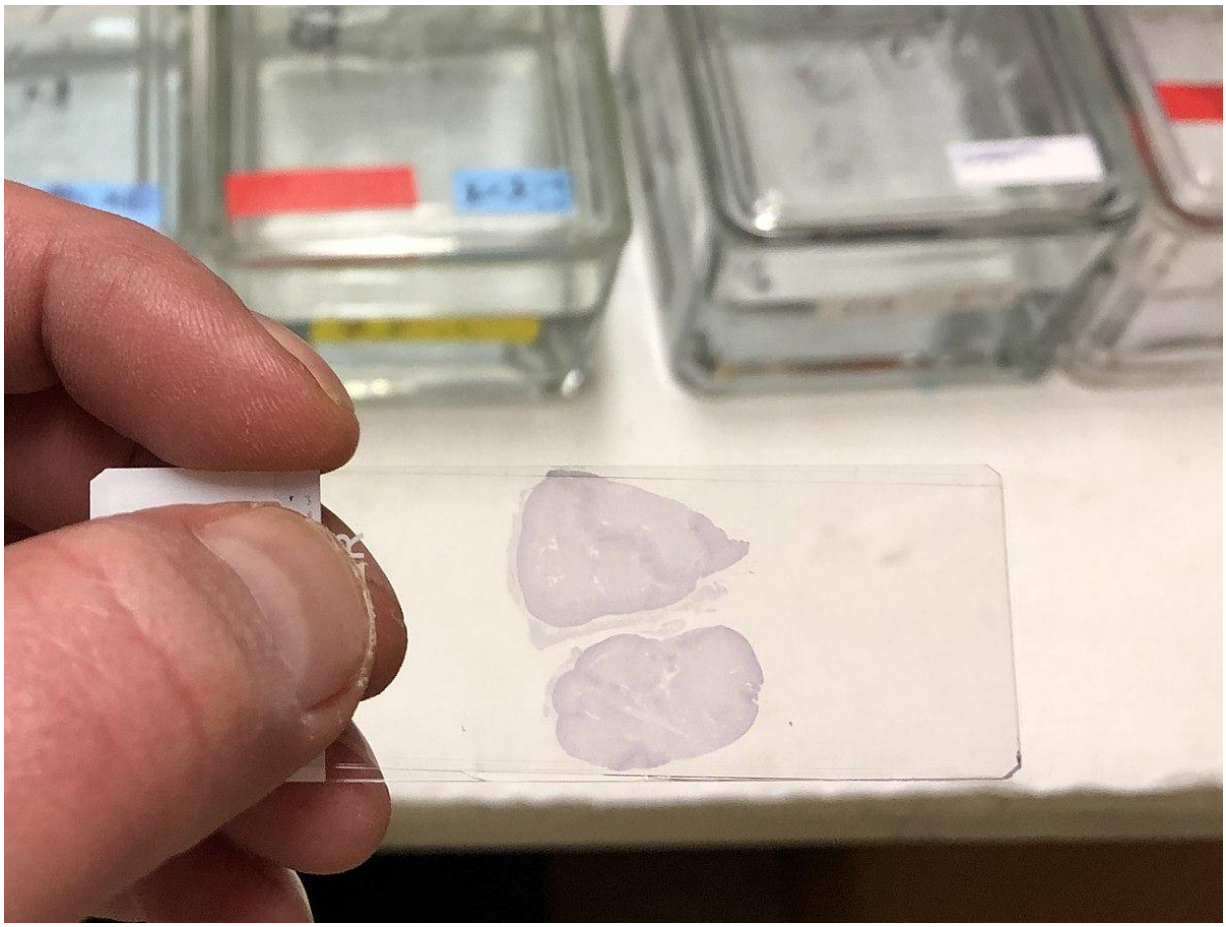


- A process called stropping produces a finely polished, smooth, and even knife edge. The knife is secured at the desired angle. place by adjusting holder screw

Section thickness and rough cutting

- A thickness of 6 µm is standard for histological tissue sections. For highly cellular tissues, 4 µm is used most often. For thicker sections, 10 µm is used. For neurological tissues and militated nerves, 6–20 and15–20 µm is used, respectively.
- The tissue block will be examined to establish how it needs to be oriented in the block holder.

- Excess paraffin should be trimmed away from each side of the tissue block to create a trapezoid shape.
- The longer edge should be parallel with the knife edge. The tissue block should be roughly cut by advancing the block manually and sectioning until the entire surface of the tissue is exposed.



Section adhesives, sectioning tissues, and sealing of blocks

- Section adhesives, such as gelatin, casein glue, starch, and albumin, can be used to aid in adhering sections to the slide prior to further processing, such as staining.
- Gelatin can be added to the water bath. The use of adhesives in the water bath promotes bacterial and fungal growth. Daily cleaning of the water bath with sodium hypochlorite solution is necessary to prevent contamination. Alternatively, a thin coat of albumin can be applied directly to the slide by dipping it into the solution or using your fifth finger.
- This latter process is referred to as “subbing.” A newer idea is to use “plus” (+) slides.

- Treatment of the slide with a reactive silicon or poly lysine compound chemically changes the glass, such that it bears abundant amino groups, which ionize to provide a positively charged surface. Sections which contain a preponderance of anionic groups, such as carboxyl's and sulfate-esters adhere strongly to this modified glass.
- When creating a ribbon (what is a ribbon), series of adjacent tissue sections, the hand wheel should be turned at a slow and even speed. Rotating the wheel too rapidly will cause sections of unequal thickness.
- The floatation bath should be heated to a few degrees below the melting point of the paraffin wax. Tap, deionized or distilled water can be used. The ribbon should be gradually lowered onto the flotation bath to eliminate wrinkles and entrapped air.
- Air bubbles may be removed with a camel’s hair brush or by submerging a slide under the ribbon. If the sections are wrinkling, a 70% alcohol solution can be added to the water bath prior to section collection.
- If necessary, sections may be separated, depending on their sizes, and each can be placed on a clean, pre-marked glass slide. Individual sections or tissue ribbons may be picked up by submerging a clean glass slide into the water bath at a ~45° angle, directly beneath the location of the section or ribbon. The slide should be lifted out of the water slowly to ensure that the sections lay on the slide.
- The slides should be drained vertically on a paper towel for several minutes before placing them onto a warming table (37–40°C). The slides should remain on the warming table, overnight, for 20–30 minutes at approximately 58°C or a few degrees below the melting point of the paraffin wax.
- Failure to drain the slides will create air bubbles under the tissue and decrease the section’s adhesion to the slide.
- Air bubbles produce section unevenness and staining artifacts, making the final preparation difficult to examine with the microscope. Once the desired sections have been cut, the block can be removed from the block holder and sealed with molten paraffin wax to ensure that the tissue will not dry out and become brittle (blocks can last for weeks, months, or years)

Problems encountered with sectioning tissue blocks

- Histologists are confronted often with difficult tissue blocks that will not section easily. This may be the result of, for example, brittle or shrunken tissue, improperly infiltrated tissues, or sections with, for example, holes or scratches in them. If the tissue block appears to be brittle, a 10% diluted ammonium hydroxide solution may be applied (via soaking) to soften the tissue to prevent cracking and to more easily facilitate sectioning.
- If sections have holes in them, this can be indicative of incompletely infiltrated tissue. This may be alleviated by placing the tissue block back in the heated wax bath to melt it and then proceed to re-embed the block.
- If art factual scratches or tears occur across the tissue sections, this may be indicative of flaws or dirt on the cutting edge of the knife and may be alleviated by repositioning or replacing the blade.
- Alternatively, other problems can occur if the tissue block appears to be too soft or too hard. If too soft, a remedy may be to place the block tissue side down on several sheets of Kim wipes or paper towel in the freezer (−15°C) or a refrigerator (0–4°C) (chilling times may vary), prior to sectioning.
- This technique will help to harden the wax so that it better matches the hardness of the infiltrated tissue and will result in more successful tissue sectioning. If too hard, a piece of wet cotton/Kim wipe may be placed in lukewarm water and then placed over the surface of the block (times may vary).
- This will allow the tissue to expand/swell and soften as it absorbs water. It should be noted, however, that with either too soft or too hard tissue blocks, these solutions are temporary and may allow only a few successful sections to be cut

Staining

- Staining of tissue slides is carried out by reversing the embedding process in order to remove the paraffin wax from the tissue to allow water-soluble dyes to penetrate the sections.
- This process is referred to as “deparaffinization.” The tissue slides must be exposed to a clearing agent and subsequently taken

through a descending alcohol series to water (also referred to as “bringing your slides to water”).

- Choosing the appropriate dye for a particular tissue slide is related to its ability to color otherwise transparent tissue sections and various cellular components of the tissue. The term “routine staining” includes the hematoxylin and eosin stain.
- This stain is used routinely as it provides the pathologist or researcher with a detailed view of the tissue, clearly staining, for example, the cytoplasm, nucleus, and organelles.
- The term “special stains” refers to a large number of staining techniques, other than H and E, that allow the visualization of particular tissue structures, elements, or microorganisms that cannot be identified with H and E staining .
- Examples include, Masson’s trachoma, GMS silver stain, Periodic acid-Schiff, such as glycogen, glycoproteins, and proteoglycans), Perl’s Prussian blue iron (Fe3+) iron in tissue preparations or blood and bone marrow smears), Ziehl-Neelsen (acid-fast bacillus) , Alcan blue, Alcan blue and PAS (intestine; combination of staining properties of both Alcan blue and Periodic acid-Schiff for identification of similar tissue components), Gomori trichromatic (blue or green), identification of muscle fibers, collagen, and nuclei.

Making and Staining a Blood Smear

A well-made blood smear is a beauty to behold, and likely to yield interesting and significant information for a research project. A poor slide is a torment. The extra time and care taken during the field season will be rewarded later when the smears must be scanned, and parasites identified and counted. Here, the methods for making and staining smears are given, as well as a list of sources for high quality slides, stain, and chemicals. Photographs showing well-made smears are shown on the website.

Dried blood samples for genetic studies should always be made at the same time as the smears. The method is very easy and modern research must combine studies of morphology under the microscope with molecular methods. The technique for making and storing dried blood samples is given in the section “Dried Blood Samples”.

Making a smear

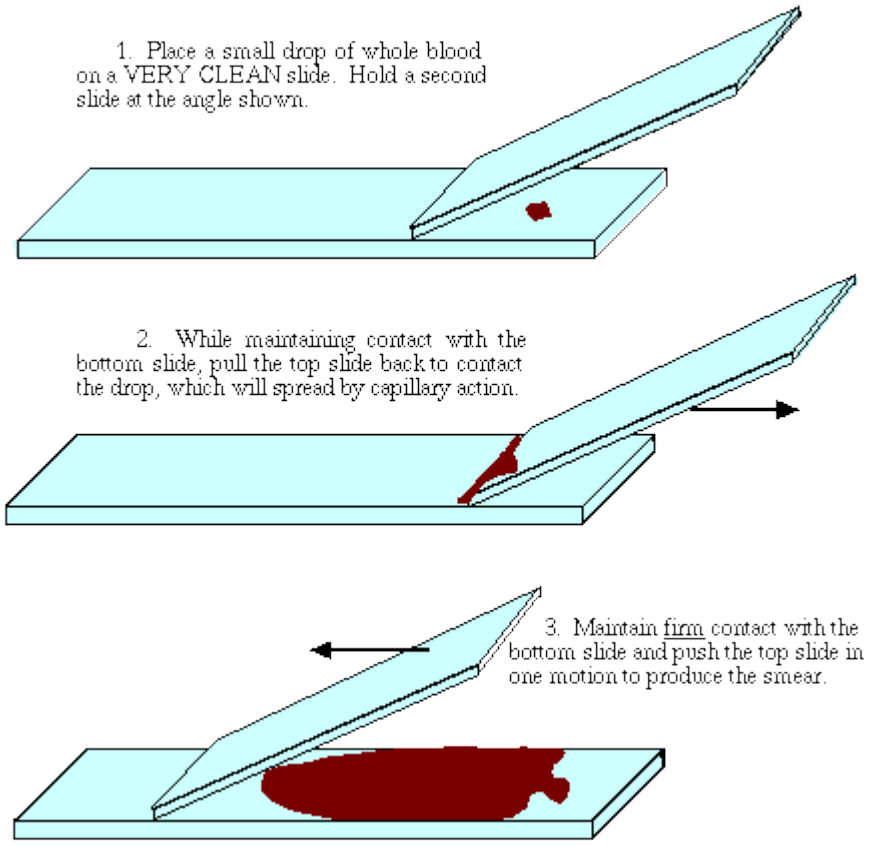
1. A single smear can be made per slide (smear running the length of the slide) or two (or even three) smears can share a slide, with the smears running the width of the slide. Putting two smears per slide saves on weight (glass is heavy) for field trips, and storage space. A picture showing both versions is included on the website.

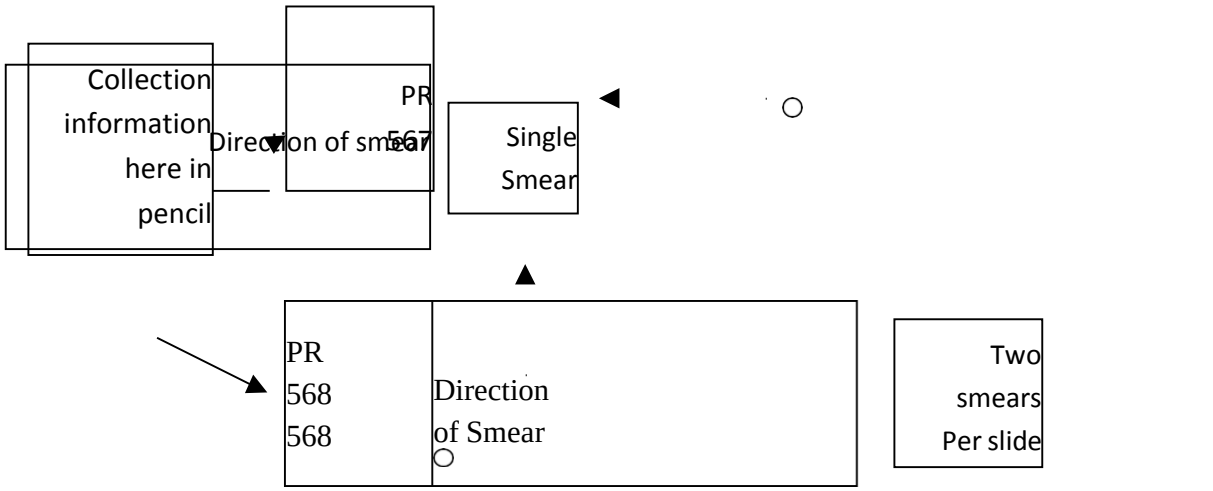
2. It is easiest to use microscope slides with a frosted end, so that identifying information can be written there with pencil. Warning: Compare different pencils to find one that does not yield labels that rub off or wash off in the methanol dip.

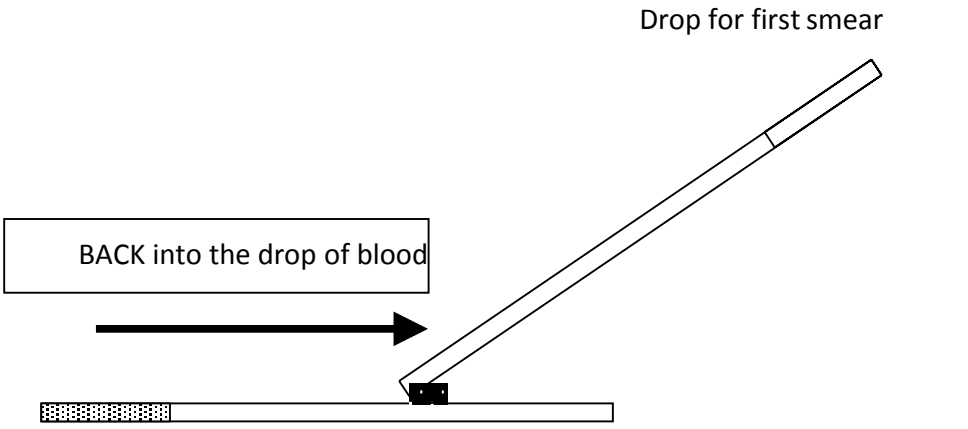
3. Place a drop of blood approximately 4 mm in diameter on the slide (near the end if one smear is to be made, or at the proper location if two smears are to share a slide). See the drawing below.

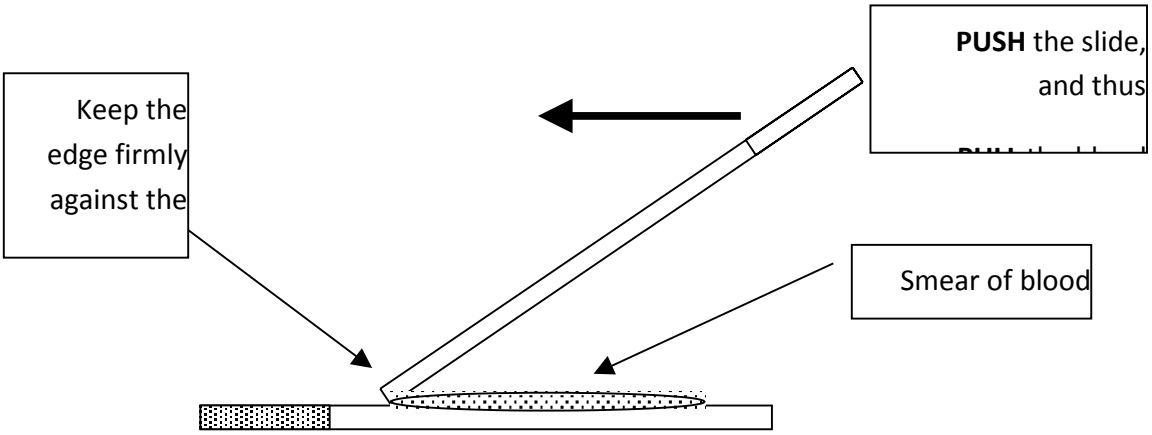
4. Spread the drop by using another slide (called here the “spreader”), placing the spreader at a 45° angle and BACKING into the drop of blood. The spreader catches the drop and it

spreads by capillary action along its edge. To make a short smear, hold the spreader at a steeper angle, and to make a longer smear, hold it closer to the drop. Now, push the spreader across the slide; this PULLS the blood across to makethe smear. Do not push the blood by having it ahead of the smearing slide! It should take about one second to smear the drop. A smooth action is required, with the edge of the spreader held against the slide. This will yield a nice, even smear.




Collection information here in pencil
Direction of smear
PR
567
Single Smear
PR
568
568
Direction of Smear
Two smears Per slide


Drop for first smear
BACK into the drop of blood


PUSH the slide, and thus
Keep the edge firmly against the
Smear of blood

5. If doing one smear per slide, the spreader then becomes the next slide to receive a smear. Thus, each slide serves two duties, as a spreader, then as a slide to receive a smear. If two smears are made per slide, be sure to flip over the spreader to use the other edge for the second smear produced. The spreader then is used to receive the next two smears. Warning: If there is surplus blood on the spreader, wipe it off carefully before flipping it over to make the second smear on the slide.

6. Photographs are shown in the website.

7. For blood taken from mammals, a THICK blood film can also be made, but this is not possible with blood from birds or reptiles. Only mammals have erythrocytes that lack a nucleus. Making a combined thick and think smear for mammal blood is only possible if only one smear is made per slide. Make the thin smear starting about 1/3 from the nonfrosted end of the slide. Then, place another drop of blood at the clear end and use the edge of the smearing slide to spread the drop out to about a 1 cm circle. The thick smear will take longer to dry. Because the erythrocytes of mammals lack a nucleus, thousands of cells can be stacked, and parasites still seen (not for identification, but simply to detect an infected animal).

8. Smears should be air-dried, and then dipped into 100% methanol. A coplin jar with a screw top is best for this. We use a plastic version, which won't break in the field, but has a poorly sealing top. Slides can be stored while drying in a small plastic slide box (holds 25 slides). Then, they are placed, two at a time, back-to-back, into the slots in the coplin jar. Thus, ten slides can be dipped at once. Be sure the alcohol does not reach the frosted end of the slide. After one minute, the slides are removed and placed on end to drain the alcohol. They can then be placed into a plastic slide box for complete drying.

9. In the field, we place the plastic slide box or boxes into a zip-

lock bag with silica gel, and they are allowed to dry overnight.

10. To store slides during long field trips, and where many slides are to be made, they can be placed back into their original cardboard boxes, with a piece of index card or other clean paper between each slide.

Field vs. lab preparation of smears (wild caught animals)

For our work with lizard malaria parasites, we always bring the lizards back into the lab in the evening for processing (even if the "lab" is a hotel room!), so the smears can be made in a somewhat controlled environment. For the work on bird parasites, smears must be made at the site of capture (usually when mist-netting in the early morning, and often in web environments). Very good quality smears are still produced by working on the tailgate of a pick-up truck, or on a field table (a piece of stiff plastic placed on the ground). Smears are kept after dipping in alcohol in a bag with silica gel. Smears made in the field in hot and dry climates often are of very poor quality, probably because they dry too rapidly. Smears made in the veterinary clinic should be of very high quality because of the uniform and clean environmental conditions.

Staining smears

1. First prepare the buffer. The stock buffer should be kept in the refrigerator, but if not possible, can be stored at room temperature for several weeks. Make working buffer which can be stored at room temperature for a few days. Buffer should be pH 7.0 to

7.2. Although this is a higher pH than normally used to stain blood cells, the parasites will stain darker and be more visible under the microscope.

2. A high-quality Giemsa should be used. Not all Giemsa stains are equal in quality. We place a layer of stain in the bottom of a glass coplin jar (about 3 mL), then add buffer to a level that will just cover the slides (except for frosted ends!) when they are in the jar. A little practice will tell the amount of buffer to add. Place the slides, back-to-back into the slots of the jar, and stain at room temperature for about 50 minutes.

3. Remove slides, rinse by dipping a few times into plain buffer, then stand on end to dry. Some workers prefer to run a thin

stream of tap water over the slide to remove all the remaining stain; we have not found this necessary. Be sure to wash out the coplin jars after each use. If not properly washed, stain builds up inside the jar and reduces the quality of staining.

4. There is no need to cover-ship the slides. Immersion oil can be placed directly on the smear for observing under 1000x.

Preparing staining buffer

Stock buffers (two)
The alkaline stock is Sodium phosphate, dibasic anhydrous, N_2HPO_4, Sigma Chemical S-0879. Mix 9.5 gm with distilled water to make 1000 mL.
The acid stock is Potassium phosphate monobasic anhydrous, KH_2PO_4, Sigma P5379, mix 9.07 gm with distilled water to make 1000 mL

Working buffer: Mix 39 mL of acid stock with 61 mL of the alkaline stock, and 900 mL of distilled water. Check pH, and adjust to ph 7 or 7.2 by adding the acid buffer stock to lower pH or alkaline to raise pH. Just a very few mL should be necessary to reach the required pH.

Other supplies

Microscope slides. Good-quality slides seldom will retain any oil from machines used in their manufacture, so cleaning should not be required. We use slides with frosted end from VWR (#48311-950).

Giemsa. Not all Giemsa stains are equal in quality. We use Baker obtained from VWR No. JTM708-1, a 500 mL bottle. This plastic bottle has a pour spout that ALWAYS leaks. So, we store the bottle in a plastic bag and always handle the bottle through the bag. Giemsa stain will color skin for several days!

Slide boxes. In the field we use blue plastic slide boxes that hold 25 slides. These are obtained from Carolina Biological Supply (Carolina Blue Boxes, #HT-63-4200)). For permanent storage, we use wooden boxes from VWR (#48450-006).

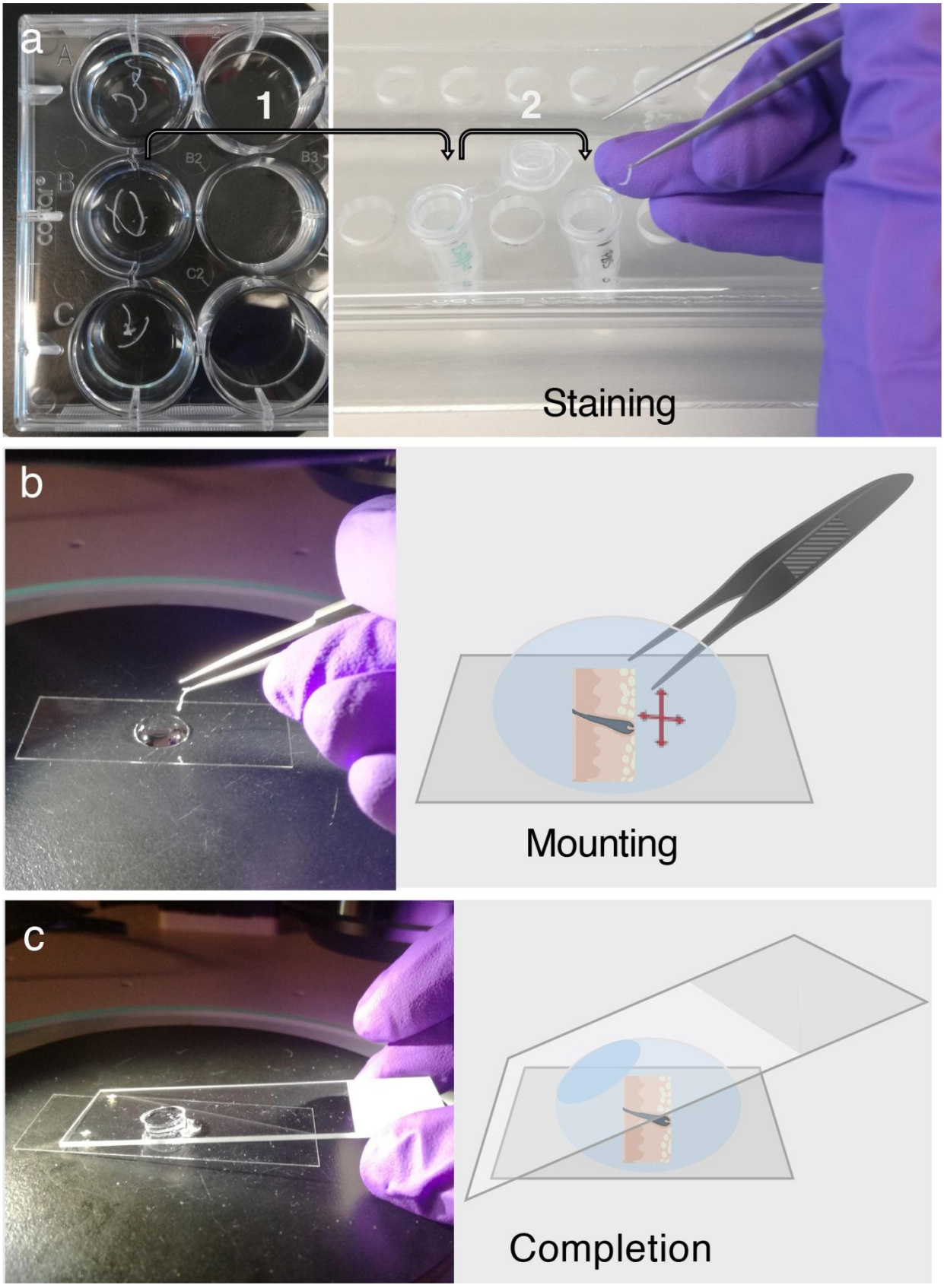
Coplin jars. The plastic jar used in the field for dipping into methanol is obtained from Carolina (#HT-74-2155). Staining jars are available from many sources (Carolina has them #HT-74-2160). Most of ours were hand-me-downs from retiring faculty over the years.

Silica gel is from Sigma (S7500) that we buy in the 1 kg can. Zip-lock plastic bags should be the ones

used for freezer storage.

Preparation of permanent slide (Mounting)

Mounting of tissue (section, squash, smear) in a suitable mounting medium (e.g. Canada balsam, Euparal). After staining, it is necessary to avoid drying up of tissue and not to render it opaque. The mounting media should have a refractive index closer to glass to avoid refraction. It should harden quickly in contact with air and should check de-staining.



The principal aims of mounting are:

(i) To render the tissue transparent;

(ii) To increase the visibility of tissue under microscope;

(iii) To hold it with the protecting coverslip firmly in place;

(iv) To preserve it for a long period.

A. Mounting of Sections and Smears after Crystal Violet Staining:

1. For dehydration, the slide is passed through absolute ethanol, keeping in each for 2 sec.
2. Differentiation is done by passing the slide through clove oil I for 2-5 min (observation under microscope is required for satisfactory staining) and then transferred to clove oil II and kept for 10-15 min.
3. For clearing, the slide is kept in xylol I, II and III for 1 hour in each.
4. Finally, it is mounted in canada balsam under a coverslip and the slide is allowed to dry overnight on a hot plate (35-45°C).

B. Mounting of Squashes/Smears after Aceto-orcein, Aceto-carmine and Feulgen staining:

(a) Acetic-alcohol schedule:

1. The paraffin seal of temporary preparations is carefully removed with blade after 1-2 days and inverted in a covered petridish containing glacial acetic acid -ethanol (1:1) mixture till the cover-glass is detached.
2. Both the slide and cover-glass with materials are transferred to ethanol and kept for 10 min.

3. These are then passed through ethanol-xylol (1:1) mixture, xylol I and xylol II, keeping in each for 10 min.
4. The slide and the cover-slip are mounted separately in canada balsam (two slides will be prepared) and allowed to dry overnight on a hot plate.

(b) Butanol schedule:

1. The paraffin seal of temporary preparations is carefully removed with blade and inverted in a covered petridish containing glacial acetic acid -ethanol (1:1) mixture till the cover-glass is detached.
2. Both the slide and cover-glass with material are transferred to ethanol: n- butyl alcohol (1:1) for 5 min.
3. Both the slide and cover-glass with material are passed through n-butyl alcohol I and n-butyl alcohol II, keeping 20-30 min in each.
4. The slide and the cover-glass are mounted separately in euparal (two slides will be prepared) and dried on a hot plate for overnight.

Another method

Quick freeze method for making slides permanent

This type of method is based on the freezing of freshly

prepared slides with dry ice. Many of use liquid nitrogen if it is available. Occasionally liquid nitrogen causes slides to crack when they are plunged into it. For preparing the slides permanent quick freeze method is highly influential. These are the following steps:

1. Freeze slide (coverslip up) on a block of dry ice.
2. Pop off the coverslip with a razor blade.
3. Immerse the slide in 95% ethanol for approximately 1 minute.
4. Remove the slide from the ethanol and cover material with a clean coverslip and either diaphane or euparal mounting resin. If a xylene- or toluene-soluble resin is to be used (e.g. permount), the slide must be passed through three changes of 100% ethanol, one change of1/1 ethanol/xylene (or toluene), and two or three changes of dry xylene (or toluene) before mounting with the resin. A limonene-based clearing agent marketed by Fisher Scientific, Hemo-De, is a suitable replacement for xylene or toluene.
5. Although slides may be examined immediately after applying a coverslip with mounting resin, the mounting resin may not become entirely hardened for several months. Use caution and a minimum amount of pressure if the coverslip must be cleaned.
6. Slides should be stored flat in a dust free environment until

the mounting resin has hardened sufficiently to prevent easy movement of the coverslip.

Protocols followed in Histotechniques

1. Identification & Labeling of the specimen
2. Fixation
3. Dehydration
4. Clearing
5. Impregnation (infiltration)
6. Embedding
7. Section cutting
8. Staining
9. Mounting

Animals should be anesthetized and subjected to cardiac perfusion with saline sol.

Fixation:

This is the process by which the constituents of cells and tissue are fixed in a physical and

chemical state so that they will withstand subsequent treatment with various reagents with

minimum loss of architecture.

This is achieved by exposing the tissue to chemical compounds: fixatives

• Fixatives prevent autolysis and bacterial decomposition and preserves tissue in their natural

state and fix all components

Tissue fixatives

• Buffered formalin (light microscope preparation)

• Buffered gluteraldehyde (electron microscope preparation)

• Osmium tetroxide (electron microscope preparation, preserve and stain)

• Zenker's formal saline

• Bowen's fluid

No fixative will penetrate a piece of tissue thicker than 1 cm

Tissue is sectioned and drop-fixed in a 10% formalin solution (24-48 hours).Formalin has an

acidic nature. Prolonged fixation of samples in formalin causes formalin pigments inside the tissue. This pigments removed by washing sections with alcohol picric acid for some minutes

The excess of formalin can be removed by

Washing samples in running tap water (24 hr.)

Washing samples in 2 changes of chloral hydrate (12 hr. for each) .

The reactions of formalin with tissue are complex because it can combine with a different groups.

Tissue Processing

In order to cut thin sections of the tissues, it should have suitable hardness and consistency when presented to the knife edge.

These properties can be imparted by infiltrating and surrounding the tissue with paraffin

wax, various types of resins or by freezing.

Tissue Processing It can be subdivided into:

a) Dehydration

b) Clearing

c) Impregnation (infiltration)

Types of tissue processing

• There are two types:

1. Manual Tissue Processing

2. Mechanical Tissue Processing.

Manual Tissue Processing

• In this process the tissue is changed from one container of reagent to another by hand

Mechanical Tissue Processing

• In this the tissue is moved from one jar to another by mechanical device

• Timings are controlled by a timer which can be adjusted in respect to hours and minutes|

• Temperature is maintained around 60 ºC.

Dehydration (removal of water) :

It is the process in which the water content in the tissue to be processed is completely

removed by passing the tissue through increasing concentrations of dehydrating agents

Tissues are dehydrated by using increasing strength of alcohol; e.g. 70% (1 hours),80%(1

hours), 90% (1 hours),95% (30 minutes) and 100% (30 minutes).

• Water is replaced by diffusion

During dehydration water in tissue has been replaced by alcohol • the next step alcohol

should be replaced by paraffin wax • as paraffin wax is not alcohol soluble, we replace

alcohol with a substance in which wax is soluble. This step is called clearing.

When samples are dehydrated by Butyl alcohol, the clearing process can be neglected.

Clearing:

• Replacing the dehydrating fluid with a fluid that is totally miscible with both the

dehydrating fluid (alcohol) and the embedding medium (wax). Clearing process is a median

stage between Alcohol and paraffin wax.

Some clearing agents: - Xylene - Toluene - Chloroform – Benzene- Methyl benzoate.

Samples are cleared in 3 changes of Methyl benzoate (24 hr. for each change).

Infiltration (Impregnation):

The tissue is kept in a wax bath containing molten paraffin wax.

Samples are infiltrated in 3 changes of paraffin wax (1 hr for each change).

Embedding:

Embedding: is the process by which impregnated tissues are surrounded by a medium such as

agar, gelatin, or wax which when solidified will provide sufficient external support during

sectioning:

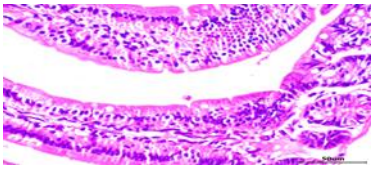
Embedding: It is done by transferring the tissue to a mould filled with molten wax & is

allowed to cool & solidify After solidification, a wax block is obtained which is then

sectioned to obtain ribbons

General Embedding Procedure.

Hematoxylin and Eosin



Ionic bonding is the most important type of bonding that occurs in histologic staining

techniques. It involves electrostatic attraction between ions of opposite charge, one of which

is fixed in the tissue, and the second of which is in the dye.

Hematoxylins alone is not technically a dye, and will not directly stain tissues. It therefore

needs to be used in combination with a “mordant” – a compound that helps it link to the

tissue. The mordant used is typically a metal cation, such as aluminium. Haematoxylin in

complex with aluminium salts is cationic and acts as a basic dye. It is positively charged and

can react with negatively charged, basophilic cell components, such as nucleic acids in the

nucleus. These stain blue as a result.

Eosin is anionic and acts as an acidic dye. It is negatively charged and can react with

positively charged, acidophilic components in the tissue, such as amino groups in proteins in

the cytoplasm.

Hematoxylins & eosin staining depends on the basic and acidic properties of tissue

components.

Hematoxylene is a basic dye, acidophilic dye

Eosin is acidic dye, basophilic dye.

The staining procedure for H&E follows a basic protocol:

- Dewaxing
- Dehydration
- Hematoxylin
- Differentiation
- Bluing
- Eosin
- Dehydration
- Clearing
- Cover-slipping

H & E Stain

Intended use

H & E Staining method is used for the routine staining of the cationic and anionic tissue components in tissue sections. This is the standard reference stain used in the study of histochemical tissue pathology.

Summary

Hematoxylin and eosin stain (abbreviated as H & E stain) is one of the principal tissue stains used in routine histology staining methods. It is the most widely used stain in medical diagnosis and is often the gold standard; wherein, when a pathologist looks at a biopsy of a suspected cancer, the histological section is likely to be stained with H & E.

H & E Stain is the combination of two histological stains: Hematoxylin and Eosin. The hematoxylin is a selective nuclear stain which stains the cell nuclei blue, and eosin stains the extracellular matrix and cytoplasm pink, with other structures taking on different shades, hues, and combinations of these colors. The stain shows the general layout and distribution of cells and provides a general overview of a tissue sample's structure. Hence, there is a clear differentiation between the nuclear and cytoplasmic parts of a cell.

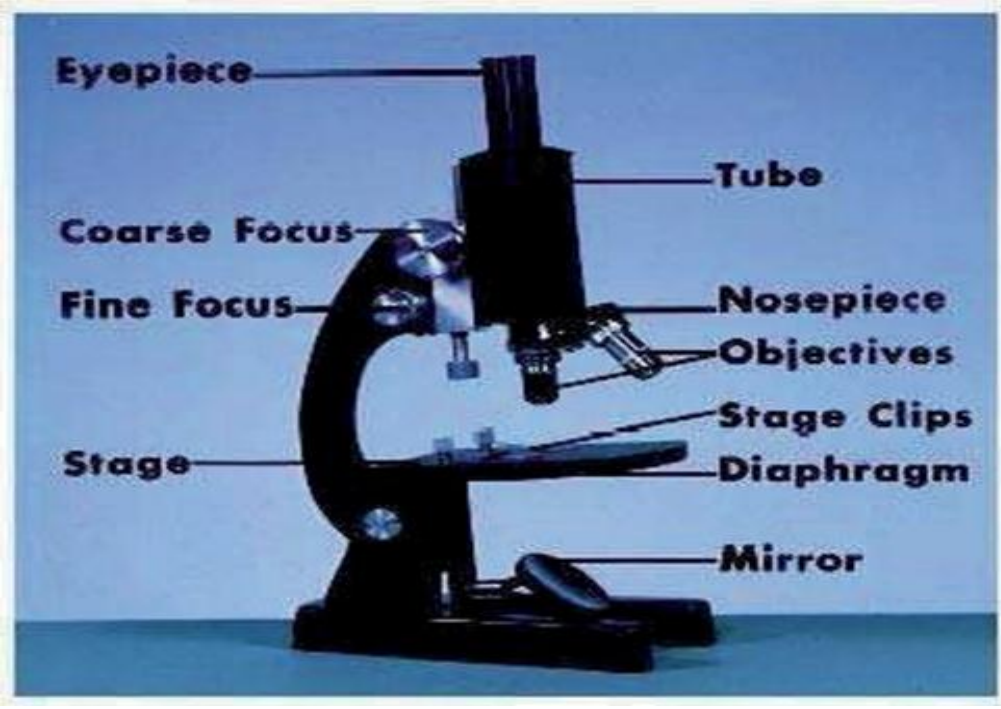
Light microscope:

A light microscope (LM) is an instrument that uses visible light and magnifying lenses to examine small objects not visible to the naked eye, or in finer detail than the naked eye allows. Magnification, however, is not the most important issue in microscopy. Mere magnification without added detail is scientifically useless, just as endlessly enlarging a small photograph may not reveal any more detail, but only larger blurs. The usefulness of any microscope is that it produces better resolution than the eye. Resolution is the ability to distinguish two objects as separate entities, rather than seeing them blurred together as a single smudge. The history of microscopy has revolved largely around technological advances that have produced better resolution.

The advancement of light microscopy:

also required methods for preserving plant and animal tissues and making their cellular details more visible, methods collectively called histotechnique (from *Histo,* meaning "tissue"). In brief, classical histotechnique involves preserving a specimen in a fixative, such as formalin, to prevent decay; embedding it in a block of paraffin and slicing it very thinly with an instrument called a microtome; removing the paraffin with a solvent; and then staining the tissue, usually with two or

more dyes. The slices of tissue, called histological sections, are typically thinner than a single cell. The colors of a prepared tissue are not natural colors, but they make the tissue's structural details more visible. A widely used stain combination called hematoxylins and eosin, for example, typically colors cell nuclei violet and the **cytoplasm** pink.



Uses of Light Microscopy

Microscopes are essential tools for scientists.

They are used in microbiology, material science, mineralogy and medicine.

A combination of staining and light microscopy can allow scientists to identify different kinds of bacteria. Staining involves adding special dyes to a smear of cells. These stains are diagnostic for different kinds of cell membranes. Gram staining, for instance, uses crystal violet to stain Gram- positive bacteria and safranin to stain Gram-negative bacteria. These will show up in the light microscope as purple Gram-positive cells and pink Gram-negative cells. Being able to identify bacteria in this way is helpful, as many Gram-negative cells are associated with infection and disease.

The optical microscope, the most common type of microscope, contains several parts with specific functions. Observe the picture and find their functions.

1. **Eyepiece**: contains the ocular lens, which provides a magnification power of 10x to 15x, usually. This is where you look through.

2. **Nosepiece**: holds the objective lenses and can be rotated easily to change magnification.

3. **Objective lenses**: usually, there are three or four objective lenses on a microscope, consisting of 4x, 10x, 40x and 100x magnification powers. In order to obtain the total magnification of an image, you need to multiply the eyepiece lens power by the objective

lens power. So, if you couple a 10x eyepiece lens with a 40x objective lens, the total magnification is of 10 x 40 = 400 times.

4. **Stage clips**: hold the slide in place.

5. **Stage**: it is a flat platform that supports the slide being analyzed.

6. **Diaphragm**: it controls the intensity and size of the cone light projected on the specimen. As a rule of thumb, the more transparent the specimen, less light is required.

7. **Light source**: it projects light upwards through the diaphragm, slide and lenses.

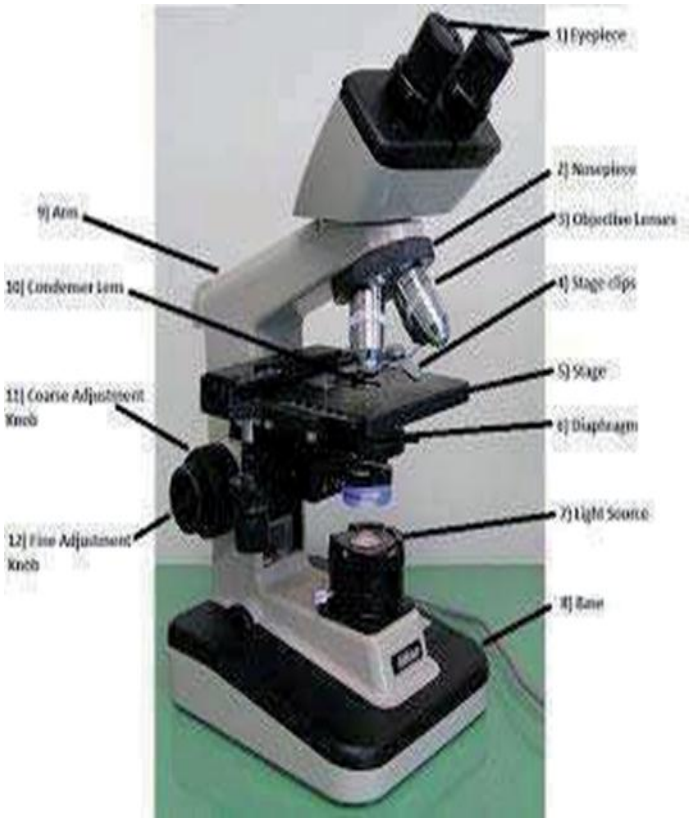
8. **Base**: supports the microscope.

9. **Condenser lens**: it helps to focus the light onto the sample analyzed. They are particularly helpful when coupled with the highest objective lens.

10. **Arm**: supports the microscope when carried.

11. **Coarse adjustment knob**: when the knob is turned, the stage moves up or down, in order to coarse adjust the focus.

Fine adjustment knob: used fine adjust the focus.



Electron microscope:
The electron microscope is a type of microscope that uses a beam of electrons to create an image of the specimen. It is capable of much higher magnifications and has a greater resolving power than a light microscope, allowing it to see much smaller objects in finer detail. They are large,

expensive pieces of equipment, generally standing alone in a small, specially designed room and requiring trained personnel to operate them.

The History of electron microscope:

By the middle of the 19th century, microscopists had accepted that it was simply not possible to resolve structures of less than half a micrometre with a light microscope because of the Abbe's formula, but the development of the cathode ray tube was literally about to change the way they looked at things; by using electrons instead of light! Hertz (1857-94) suggested that cathode rays were a form of wave motion and Weichert, in 1899, found that these rays could be concentrated into a small spot by the use of an axial magnetic field produced by a long solenoid. But it was not until 1926 that Busch showed theoretically that a short solenoid converges a beam of electrons in the same way that glass can converge the light of the sun, that a direct comparison was made between light and electron beams. Busch should probably therefore be known as the father of electron optics. In 1931 the German engineers Ernst Ruska and Maximillian Knoll succeeded in magnifying and electron image. This was, in retrospect, the moment of the invention of the electron microscope but the first prototype was

actually built by Ruska in 1933 and was capable of resolving to 50 nm. Although it was primitive and not really fit for practical use, Ruska was recognised some 50 years later by the award of a Nobel Prize. The first commercially available electron microscope was built in England by Metropolitan Vickers for Imperial College, London, and was called the EM1, though it never surpassed the resolution of a good optical microscope. The early electron microscopes did not excite the optical microscopists because the electron beam, which had a very high current density, was concentrated into a very small area and was very hot and therefore charred any non-metallic specimens that were examined. When it was found that you could successfully examine biological specimens in the electron microscope after treating them with osmium and cutting very thin slices of the sample, the electron microscope began to appear as a viable proposition. At the University of Toronto, in 1938, Eli Franklin Burton and students Cecil Hall, James Hillier and Albert Prebus constructed the first electron microscope

in the New World. This was an effective, high- resolution instrument, the design of which eventually led to what was to become known as the RCA (Radio Corporation of America) range of very successful microscopes.

How electron microscopes work

If you've ever used an ordinary microscope, you'll know the basic idea is simple. There's a light at the bottom that shines upward through a thin slice of the specimen. You look through an eyepiece and a powerful lens to see a considerably magnified image of the specimen (typically 10–200 times bigger). So there are essentially four important parts to an ordinary microscope:

1. The source of light.
2. The specimen.
3. The lenses that makes the specimen seem bigger.
4. The magnified image of the specimen that you see.

In an electron microscope, these four things are slightly different.

1. The light source is replaced by a beam of very fast moving electrons.
2. The specimen usually has to be specially prepared and held inside a vacuum chamber from which the air has been pumped out (because electrons do not travel very far in air).

3. The lenses are replaced by a series of coil-shaped electromagnets through which the electron beam travels. In an ordinary microscope, the glass lenses bend (or refract) the light beams passing through them to produce magnification. In an electron microscope, the coils bend the electron beams the same way.

4. The image is formed as a photograph (called an **electron micrograph**) or as an image on a TV screen.

That's the basic, general idea of an electron microscope. But there are actually quite a few different types of electron microscopes and they all work in different ways. The three most familiar types are called transmission electron microscopes

(TEMs), scanning electron microscopes (SEMs), and scanning tunneling microscopes (STMs).

Transmission electron microscopes (TEMs)

A TEM has a lot in common with an ordinary optical microscope. You have to prepare a thin slice of the specimen quite carefully (it's a fairly laborious process) and sit it in a vacuum chamber in the middle of the machine. When you've done that, you fire an electron beam down through the specimen from a giant electron gun at the top. The gun uses electromagnetic coils and high voltages (typically from 50,000 to several million volts) to accelerate the electrons to very high speeds. Thanks to our old friend wave-particle duality, electrons (which we normally think of as particles) can behave like waves (just as waves of light can behave like particles). The faster they travel, the smaller the waves they form and the more detailed the images they show up. Having reached top speed, the electrons zoom through the specimen and out the other side.

where more coils focus them to form an image on screen (for immediate viewing) or on a photographic plate (for making a permanent record of the image). TEMs are the most powerful electron microscopes: we can use them to see things just 1 nanometer in size, so they effectively magnify by a million times or more.

Scanning electron microscopes (SEMs)

Most of the funky electron microscope images you see in books—things like wasps holding microchips in their mouths—are not made by TEMs but by scanning electron microscopes (SEMs), which are designed to make images of the surfaces of tiny objects. Just as in a TEM, the top of a SEM is a powerful electron gun that shoots an electron beam down at the specimen. A series of electromagnetic coils pull the beam back and forth, scanning it slowly and systematically across the specimen's surface. Instead of traveling through the specimen, the electron beam effectively bounces straight off it. The electrons that are reflected off the specimen (known

as secondary electrons) are directed at a screen, similar to a cathode-ray TV screen, where they create a TV-like picture. SEMs are generally about 10 times less powerful than TEMs (so we can use them to see things about 10 nanometers in size). On the plus side, they produce very sharp, 3D images (compared to the flat images produced by TEMs) and their specimens need less preparation.

Scanning tunneling microscopes (STMs)

Among the newest electron microscopes, STMs were invented by Gerd Binnig and Heinrich Rohrer in 1981. Unlike TEMs, which produce images of the insides of materials, and SEMs, which show up 3D surfaces, STMs are designed to make detailed images of the atoms or molecules on the surface of something like a crystal. They work differently to TEMs and SEMs too: they have an extremely sharp metallic probe that scans back and forth across the surface of the specimen. As it does so, electrons try to wriggle out of the specimen and jump across the gap, into the probe, by an unusual phenomenon called "tunneling". The closer the probe is to the surface, the easier it is for electrons to tunnel into it, the more electrons escape, and the greater the tunneling current. The microscope constantly moves the probe up or down by tiny amounts to keep the tunneling current constant. By recording how much the probe has to move, it effectively measures the peaks and troughs of the specimen's surface. A computer turns this information into a map of the specimen that shows up its detailed atomic structure. One big drawback of ordinary electron microscopes is that they produce amazing detail using high-energy beams of electrons, which tend to damage the objects they're imaging. STMs avoid this problem by using much lower energies.

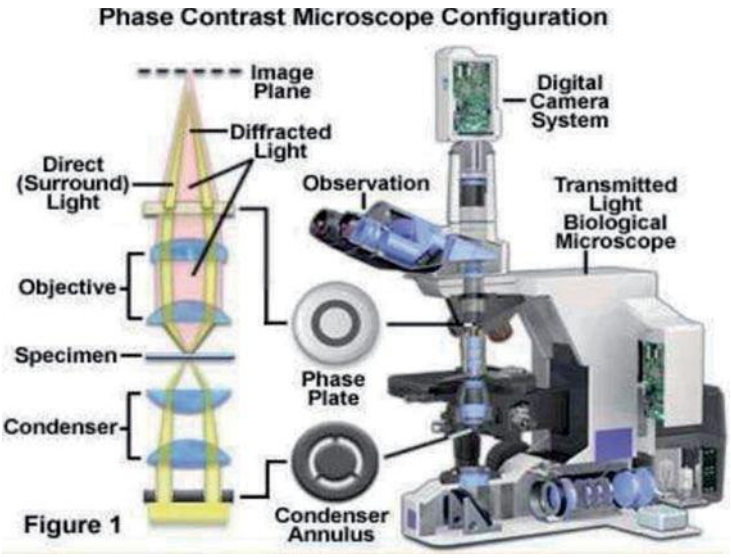
Dissecting microscope:

Dissecting microscopes, which is also called as stereoscope are stereomicroscope that are often use on acquiring a 3D view on a specimen. From the name itself, it is usually applicable in dissection of specimens, but they still have other function.

It has two eyepiece which links the two lens arrays that are arranged to established a stereoscopic image. This allows the researcher to see the 3D object on the platform of microscope clearly. The stage of a dissecting microscope is usually big with a hollow for securing the specimens being examined.

The magnification of this microscope is commonly less than a hundred times and it is lower compare to the compound microscope. This magnification feature can be settle.

Phase contrast microscope



With a conventional biological microscope, it is difficult to observe colorless, transparent cells while they are alive. A phase contrast microscope makes it possible by utilizing two characteristics of light, diffraction and interference, to visualize specimens based on brightness differences (contrast). Principle:

With regard to periodic movements, such as sinusoidal waves, the phase represents the portion of the wave that has elapsed relative to the origin. Light is also an oscillation and the phase changes, when passing through an object, between the light that has passed through (diffracted light) and the remaining light (direct light). Even if the object is colorless and transparent, there is still a change in phase when light pass through it. This phase contrast is converted into brightness differences to observe specimens.

Features:

- Transparent cells can be observed without staining them because the phase contrast can be converted into brightness differences.
- Because it is not necessary to stain cells, cell division and other processes can be observed in a living state.

Structure:

Because diffracted light is too weak to be normally observed by the eye, a phase plate is located at the focal point of light between the objective lens and the image surface so that only the phase of the direct light changes. This generates contrast on the image surface.

Structural features include a ring aperture, instead of a pinhole, on the focal plane of the converging lens and a phase plate on the rear focal plane of the objective lens.

Dark field microscope:

Most people who have survived a biology class know what a light field microscope is. This type of scope uses bright field illumination, meaning it floods the specimen with white light from the condenser without any interference. Thus the specimen shows up as a dark image on a light background (or white field if you will).

This type of unit works best with specimens that have natural color pigments. The samples need to be thick enough to absorb the incoming light; so staining is usually paired with this type of microscope.

Yet what if the specimen is light colored or translucent, like the plankton on the right? It certainly won't stand out against a strong white background. Additionally, some specimens are just too thin. They cannot absorb any of the light that

passes through them, so they appear invisible to the user. This is where the concept of dark field illumination comes in.

Rather than using direct light from the condenser, one uses an opaque disk to block the light into just a few scattered beams. Now the background is dark, and the sample reflects the light of the beams only. This results in a light colored specimen against a dark background (dark field), perfect for viewing clear or translucent details.

On a grand scale, the same thing happens every day when you look up at the sky. Do the stars disappear when it's light out? Of course not! They're still there, their brilliance blotted out by the mid-day sun.

Dark field microscopes are used in a number of different ways to view a variety of specimens that are hard to see in a light field unit. Live bacteria, for example, are best viewed with this type of microscope, as these organisms are very transparent when unstained.

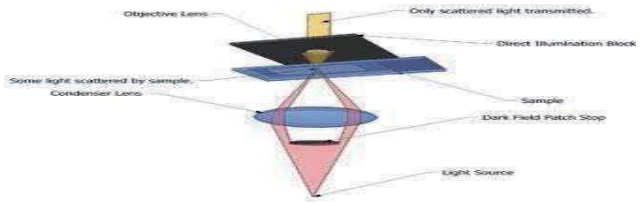
There are multitudes of other ways to use dark field illumination, often when the specimen is clear or translucent. Some examples:

- Living or lightly stained transparent specimens
- Single-celled organisms
- Live blood samples
- Aquatic environment samples (from seawater to pond water)
- Living bacteria
- Hay or soil samples

- Pollen samples

- Certain molecules such as caffeine crystals

Dark field microscopy makes many invisible.



Fluorescent Microscope

A fluorescence microscope is much the same as a conventional light microscope with added features to enhance its capabilities.

- The conventional microscope uses visible light (400-700 nanometers) to illuminate and produce a magnified image of a sample.

- A fluorescence microscope, on the other hand, uses a much higher intensity light source which excites a fluorescent species in a sample of interest. This fluorescent species in turn emits a lower energy light of a longer wavelength that produces the magnified image instead of the original light source.

Fluorescent microscopy is often used to image specific features of small specimens such as microbes. It is also used to visually enhance 3-D features at small scales. This can be accomplished by attaching fluorescent tags to anti-bodies that in turn attach to targeted features, or by staining in a

less specific manner. When the reflected light and background fluorescence is filtered in this type of microscopy the targeted parts of a given sample can be imaged. This gives an investigator the ability to visualize desired organelles or unique surface features of a sample of interest. Confocal fluorescent microscopy is most often used to accentuate the 3-D nature of samples. This is achieved by using powerful light sources, such as lasers, that can be focused to a pinpoint. This focusing is done repeatedly throughout one level of a specimen after another. Most often an image reconstruction program pieces the multi-level image data together into a 3-D reconstruction of the targeted sample.

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