Cytological &histological Technique L1 Dr. Muna Al- Rubiae

Cell: Is the functional basic unit of life. It was discovered by Robert Hooke and is the functional unit of all known living organisms. It is the smallest unit of life that is classified as a living thing, and is often called the building block of life.

Cytology: Is that branch of life science, which deals with the study of cells in terms of structure, function and chemistry.

Histology: Is the study of the microscopic anatomy of cells and tissues of plants and animals. It is performed by examining a thin slice (section) of tissue under a light microscope or electron microscope.

Histopathology: Refers to the microscopic examination of tissue in order to study the diseased tissue. Specifically, in clinical medicine, histopathology refers to the examination of a biopsy or surgical specimen by a pathologist, after the specimen has been processed and histological sections have been placed onto glass slides.

Microtechnique: Methods used in preparing microscopic sections from organ, blood smear or from different tissues.

Biopsy: Small portions of tissue are removed surgically from the living subject for histological examination.

Autopsy: The removal and examination of the internal organs of dead persons, also known as a post-mortem examination. Autopsies are either performed for legal or medical purposes.

Collection of tissues: Histopathological examination of tissues starts with surgery, biopsy or autopsy. The tissue is removed from the body or plant, and then placed in a fixative which stabilizes the tissues to prevent the degradation. The most common fixative is formalin (10% formaldehyde in water).

Introduction

Histological techniques are processes done on tissues which are collected from human or animal bodies.

There are two approaches available for the investigation of the structure of tissues, namely the direct observation of the living cells and the making of permanent preparation of dead cells.

Certain cells and tissues may be examined as soon as they are removed from the body this is the case with blood, lymph, smears from the spleen, scraping from the uterus and connective tissue.

Examination of fresh tissues has the great advantage of yielding information as to structure in conditions most closely resembling those of the body; it may be also a method of great value where speed of preparation is essential for diagnosis. On the other hand, the preparation is not permanent, and they rapidly damage unless subsequently fixed.

Permanent preparation have the advantage of providing a record of structure that unchanged for years, in addition, the fixation necessary for such preparation make it possible to apply a great variety of histological processes to the tissue, thus allowing of a very complete investigation.

Some type of cells can be satisfactorily studied by placing them directly on slides for staining and for microscopic observation (e.g. blood smears). However, for most cytological work it is necessary to cut tissues into thin, translucent slices only a few microns thick.

Sectioning is done on instruments called microtomes and is facilitated by freezing the tissue or by embedding it in a supporting medium such as paraffin or plastics.

Fixation

Most commonly, the first step in the preparation of histological material is fixation. In order to provide permanent preparations the fresh tissue must be fixed.

Fixation is a process by which biological tissues are preserved from decomposition, either through autolysis or bacterial decomposition, and maintain the structure of the cell and of sub-cellular components such as cell organelles (e.g., endoplasmic reticulum, mitochondria).

Autolysis is self destruction and is caused after the death of cells by the action of intracellular enzymes whose normal behavior is changed causing the breakdown of protein and liquefaction of cells.

Bacterial decomposition causes changes in the tissue that are very similar to those of autolysis and is caused by presence of multiplying bacteria in the diseases tissue at time of death or by bacteria normally present in the body in life such as the non-pathogenic organisms in the intestine.

Purpose of fixation

Fixation preserves a sample of biological material (tissue or cells) as close to its natural state as possible in the process of preparing tissue for examination. Fixation stabilizes tissue, preventing post-mortem change, begins a hardening which facilitates sectioning, and promotes affinity of certain tissue elements for particular dyes.

The best results are obtained by putting tissues into fixative as soon as possible after sample collection. If this is not possible, they should be

placed in the refrigerator which slows both autolysis and bacterial decomposition.

Fixation process

Fixation is the process of treating pieces of tissue with certain fluids called fixative. Fixation is usually the first stage in a multistep process to prepare a sample of biological material for microscopy or other analysis. Therefore, the choice of fixative and fixation protocol may depend on the additional processing steps and final analyses that are planned. For example, immunohistochemistry uses antibodies that bind to a specific protein target. Prolonged fixation can chemically mask these targets and prevent antibody binding. In these cases, a 'quick fix' method using cold formalin for around 24 hours is typically used. For electron microscopy, the most commonly used fixative is glutaraldehyde, usually as a 2.5% solution in phosphate buffered saline. In the process of fixation, proteins are cross-linked or denatured and rendered insoluble; lipids and carbohydrates may or may not be preserved, depending on the nature of the fixative. For example, many fats are removed from tissues immersed in alcohols. Therefore, it has often been necessary to use different technical procedures to study the various constituents of a cell.

Factors Affecting Fixation

1- **pH:** should be kept in the physiological range, between pH 4-9. The pH for the ultrastructure preservation should be buffered between pH 7.2 to 7.4.

2- **Osmolarity:** Hypotonic solutions give rise to cell swelling. Hypertonic solutions result in cell shrinkage and poor fixation.

3- **Size of the Specimen:** 1-4mm Thickness.

4- **Volume of the Fixative:** At least 15-20 times greater than tissue volume.
5- **Temperature:** Increasing the temperature increases speed of fixation. However, care is required to avoid cooking the specimen.
6- **Duration:** As a general rule 1hr per 1mm.

Chemical Fixation

In this process, structures are preserved in a state (both chemically and structurally) as close to living tissue as possible. This requires a chemical fixative that can stabilize the proteins, nucleic acids and mucosubstances of the tissue by making them insoluble.

The sample of tissue is immersed in fixative of volume at a minimum of 20 times greater than the volume of the tissue to be fixed. The fixative must diffuse through the tissue to fix, so tissue size and density, as well as type of fixative must be considered. Using a larger sample means it takes longer time for the fixative to reach the deeper tissue.

Requirements in a fixative

-Rapid penetration.
-Production of immediate death of cells.

-Stabilization of structure against further treatments.

Routine chemical fixative

1- Formalin mixtures.
   a- Formal saline, 10 percent in isotonic NaCl solution (or in phosphate buffer). Suitable for all tissues, preserves lipid.
   b- In combination with Muller's fluid (Potassium dichromate 2.5 gm, sodium sulphate1 gm, DW 100 ml).
2- Alcohol mixtures.
   a- 96 percent or absolute alcohol. Suitable for films or preserving granules and bacteria. Causes shrinkage due to dehydration.

3- Chromic acid mixtures.
   a- Muller’s fluid. Suitable for large pieces of brain or cord.
4- Mercuric chloride mixtures.
   a- Zenker's fluid.
      Mercuric chloride 5 gm, potassium dichromate 2.5 gm, sodium sulphate 1 gm, distilled water 100 ml. Suitable for microanatomy, but mercury salt precipitate must be removed by treatment with iodine in potassium iodide before staining.
5- Osmium tetroxide mixtures.
   a- Osmium tetroxide, 1 percent. Excellent for granules and nuclei. Penetrates very slowly. Much used for electron microscopy.
   b- Marchi's fluid. (Muller's fluid 20 ml, Osmium tetroxide solution 1 percent 10 ml).
6- Picric acid mixtures.
   a- Bouin's fluid. Picric acid 75 ml, 40% formaldehyde25 ml, glacial acetic acid 5 ml. Suitable for most tissues, which it fixes rapidly. Remove by washing in alcohol, not water.

Special fixation methods

Frozen section fixation

Frozen section is a rapid way to fix and prepare histology sections. It is used in surgical removal of tumors, and allows rapid determination of diseases. It is done using a refrigeration device called a cryostat. The frozen tissue is sliced using a microtome, and the frozen slices are placed on a glass slide and stained the same way as other methods.

Freeze drying technique

The freeze drying technique seems to cause less alteration of the living tissue than do the standard methods. In this technique, fresh tissue is preserved by placing it in a liquid such as isopentane chilled to about -170 °C with liquid nitrogen. The frozen tissue is dehydrated in a vacuum and may thus be embedded without previous chemical fixation and dehydration in alcohols. This method is particularly useful for studying the localization of certain enzymes which are destroyed by the standard methods.

Washing

After fixation, one must usually wash out the fixative. It cannot be left within the tissue, since its presence typically interferes with future steps in the preparation of the tissue and slides. This step of washing the tissue can be very important. Thorough removal of fixative may be important. Also, if the fixative had a high alcohol percentage, washing in a dilute solution would be a mistake since rapid water entry into the tissue will cause damage. Typically, one washes the tissue in a series of solutions to fully extract the fixative, taking enough time in each wash to fully replace the tissue's fluids. The washes may be in water or in some particular percentage of alcohol.

Tissue fixed in zenker's fixative, after fixation, the tissue must be wash with running tap water (12-24 hr) because precipitate protein difficult to remove by tap water. The tissue fixed in fixative contain picric acid, the tissue must be wash with 70% alcohol (for 3-8 hr.) but not in tap water.

Dehydration

For sectioning, the tissue is commonly infiltrated with paraffin. Because paraffin does not mix with water, wet fixed tissues (in aqueous solutions) cannot be directly infiltrated with paraffin. The paraffin will not penetrate into tissue until the water is removed. The water from the tissues must be removed by dehydration. Dehydration is usually done with dehydrant which is a substance miscible with water and capable to replace it. The commonly used dehydrant is a series of alcohols; 70% to

95% to 100%. The volume of the dehydrant should be 50-100 times greater than tissue specimens. Sometimes the first step is a mixture of formalin and alcohol. Other dehydrants can be used, but have major disadvantages. Acetone is very fast, but a fire hazard, so is safe only for small, hand-processed sets of tissues. Dioxane can be used without clearing, but has toxic fumes. Now, because paraffin is also insoluble in alcohol, the latter must be replaced by an agent miscible with both alcohol and paraffin e.g., xylene or cedar wood oil.

Dehydration may take a few steps. One cannot subject their tissue to a change from water to 100% alcohol in one quick change... tissue damage would be extensive, and usually show high degree of shrinkage due to the rapid removal of the water. Therefore, one typically uses a series of gradual changes, moving tissue through: water, 30% EtOH, 50% EtOH, 70% EtOH, 80% EtOH, 95% EtOH, 100% EtOH. The minimum duration of treatment in graded alcohol will depend on the size, type of tissue. Other alcohols can be used for dehydration rather than just ethanol (e.g., isopropyl alcohol, butyl alcohols, and methanol), but the standard (and the one that seems to work best) is ethanol; the other alcohols may have negative effects when one reaches the next steps for embedding.

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Clearing

The next step is called "clearing" and consists of removal of the dehydrant with a substance that will be miscible with the embedding medium (paraffin). Paraffin is not soluble in ethanol (or any of the other alcohols mentioned). It is, however, soluble in xylene and in toluene. Therefore, the tissue will have to be transferred into xylene before embedding can begin. This step is also called clearing the tissue, since immersion in xylene causes the tissue to become transparent. (Note: Xylene also hardens the tissue, making it a brittle). The commonest clearing agent is xylene. Toluene works well, and is more tolerant of small amounts of water left in the tissues, but is 3 times more expensive than xylene. Chloroform is also used in clearing, but is a health hazard, and is slow. Methyl salicylate is rarely used because it is expensive, but it smells nice (it is oil of wintergreen).

The steps for embedding in paraffin include the following solution changes: 100% xylene, 100% xylene, 1:1 100% xylene : paraffin, pure paraffin, pure paraffin. Once paraffin is included in the solution changes, the tissue and solution must remain inside a paraffin oven so that it remains a liquid. Also, all of the initial solution changes should take place in glass containers, since xylene dissolves plastic. Once the tissue is finished in the second pure paraffin solution, it is ready for its final step for embedding.

The time taken for complete clearing depends on the agent used and also on the size and thickness of the piece.

Clearing agents

1- Xylene

-Highly inflammable

- Rapid in action

- Soft brain, spleen become brittle

- Not toxic

- May cause dermatitis

- Cause shrinkage of delicate tissue

2- Benzene

- Similar to the xylene but it does not cause soft tissue brittle and it is toxic in low concentration.

3-Toluene

- Similar to the xylene and benzene but it is less toxic, causes little hardening.

4- Chloroform

- Not inflammable
- Slow in action
- Toxic vapour
- Little hardening effect so used for brain.

5- Cedar wood oil

- Toxic

- Very slow in action
- Suitable for large piece as brain, lymph gland.

Clearing in xylene or benzene should take half to three hours, prolong treatment in xylene or benzene causes brittleness of the tissue with subsequent difficulty in cutting.

Sometime the xylene or benzene takes shows a milky appearance when added to the tube containing the tissue. This indicated that dehydration has not been completely carried out and the tissue must therefore be put into a fresh supply of absolute alcohol for a farther period of time. If the tissue cleared quickly, the clearing agent does not reach the center.

Infiltration & Embedding

The tissue must be processed into a form in which it can be made into thin microscopic sections, the usual way this is done with paraffin, which replaces the clearing agent; this step is referred to as infiltration. Next, the tissue is embedded in paraffin by allowing the latter to harden, and then the material is ready for sectioning and subsequent staining. Tissues embedded in paraffin, which is similar in density to tissue, can be sectioned at anywhere from 3 to 10 microns, usually 6-8 routinely.

Embedding **–** Is the casting or blocking of tissue section, which involves the enclosure of the tissue in the infiltration medium used for processing, and then allowing the medium to solidify. The infiltrating medium is selected according to the embedding media that will be used.

Infiltrating and embedding media must fill all spaces within the tissue to support cellular components adequately during microtomy. Density of the hardened medium should approach that of the densest tissue component otherwise section deformation will result.

Paraffins can be purchased that differ in melting point, for various hardnesses, depending upon the way the histotechnologist likes them and upon the climate (warm vs. cold). A product called paraplast contains added plasticizers that make the paraffin blocks easier for some technicians to cut. A vacuum can be applied inside the tissue processor to assist penetration of the embedding agent.

If the tissue (which is totally transparent) is just dropped into the chamber and then the paraffin is allowed to harden, it will be impossible to find the tissue within the paraffin block for proper sectioning. Therefore, the chamber must be kept warm (by placing it on a warming plate) so that the tissue can be properly oriented. After dropping the tissue into the chamber, warm forceps can be used to

manipulate the tissue into a specific location and orientation within the chamber. The chamber can then be marked so that the tissue will be easy to locate after the paraffin cools.

The wax is kept at melting point 50-60 °C this temperature in a thermostatically controlled bath. Give two changes of wax, with a total stay of about three hours in the bath. Two or three changes of paraffin are employed to reduce traces of the clearing agent, which soften the paraffin and make sectioning of tissue difficult. Since prolonged treatment in molten paraffin cause shrinkage and hardening of the tissue they should be left in this medium no longer than necessary.

Duration of the bath depends on the size, thickness, density, and nature of the specimen. Skin and nervous tissue infiltrate slowly with paraffin. Muscle, fibrous tissue, blood will become over hardened in paraffin if left in this medium for more than three hours. Tissue from the brain and spinal cord, due to their compact nature, needs relatively longer treatment in paraffin and requires 4 to 6 hours infiltration for medium sized section.

Tissues are embedded by placing them in a template filled with melted embedding medium which is then allowed to solidify.

1- Make a cell of suitable size from two L-shaped metal blocks on a surface of glass.
2- Select the template, there should be sufficient room for the tissue with allowance for at least a 2 mm surrounding margin of wax.
3- Fill the template with paraffin wax.
4- Using warm forceps select the tissue, taking care that it does not cool in the air; at the same time.
5- Place the tissue in the template according to the side to be sectioned. This side should be facing down against the template. A small amount of pressure may be used in order to have more even embedding.
6- Chill the template on the cold plate, orienting the tissue and firming it into the wax with warmed forceps. This ensures that the correct orientation is maintained and the tissue surface to be sectioned is kept flat.

7- Cool the block on the cold plate.

8- Remove the block from the template.

9- Cross check block, label and worksheet.

Leave block in cold water for at least two hours. Slow cooling would allow large crystals to form so fragmentation would be during section cutting. Excessive cooling (ice water) produces a similar effect; Ordinary cold tap water (about 10- 12 °C) gives satisfactory results.

Celloidin is an alternative embedding medium which is particularly useful for cutting large objects (e.g., brain) and for hard and brittle material (e.g., cartilage). Plastics, particularly epoxy resins, are increasingly being employed as the embedding medium for both light and electron microscopy. They produce less tissue and cellular damage than paraffin and allow the production of much thinner sections (down to 0.02 μm).

Alternatives to paraffin embedding include various plastics that allow thinner sections. Such plastics include methyl methacrylate, glycol methacrylate, araldite, and epon. Methyl methacrylate is very hard and therefore good for embedding undecalcified bone. Glycol methacrylate has the most widespread use since it is the easiest to work with. Araldite is about the same as methacrylate, but requires a more complex embedding process. Epon is routinely used for electron microscopy where very thin sections are required.

Plastics require special reagents for dehydration and clearing that are expensive. For this reason, and because few tissues are plastic embedded, the processing is usually done by hand. A special microtome is required for sectioning these blocks. Small blocks must be made, so the technique lends itself to small biopsies, such as bone marrow or liver.

Embedding can also be accomplished using frozen, non-fixed tissue in a water-based medium. Pre-frozen tissues are placed into molds with the liquid embedding material, usually a water-based glycol, Cryogel, which is then frozen to form hardened blocks.

Sectioning

Once the tissues have been embedded, they must be cut into sections that can be placed on a slide. Sectioning is the production of very thin slices from a tissue sample. The tool used for sectioning is called a microtome. The microtome is nothing more than a knife with a mechanism for advancing a paraffin block standard distances across it.

Most laboratory microtomes have the essential machinery of a slicer:

- Cutting edge (which may be a heavy knife, a piece of broken glass, or a finely sharpened diamond).
- Specimen holder.
- Screw to advance the specimen toward the knife.

Microtomes have a mechanism for advancing the block across the knife. Usually this distance can be set, for most paraffin embedded tissues at 6 to 8 microns.

Sections for routine light microscopy are typically 5-10µm (micrometers, microns) in thickness. Exceptionally thin sections may less than 2µm thick. For electron microscopy, sections are typically 50-100 nanometers (millimicrons) in thickness.

For light microscopy, a steel knife mounted in a microtome is used to cut 10-micrometer-thick tissue sections which are mounted on a glass microscope slide. For transmission electron microscopy, a diamond knife mounted in an ultramicrotome is used to cut 50-nanometer-thick tissue sections which are mounted on a 3-millimeter-diameter copper grid. Then the mounted sections are treated with the appropriate stain.

Plastic blocks (methacrylate, araldite, or epon) are sectioned with glass or diamond knives. A glass knife can section down to about 1 micron. Thin sections for electron microscopy (1/4 micron) are best done with a diamond knife which is very expensive.

Once sections are cut, they are floated on a warm water bath that helps remove wrinkles. Then they are picked up on a glass microscopic slide.

The glass slides are then placed in a warm oven for about 15 minutes to help the section adhere to the slide. If this heat might harm such things as antigens for immunostaining, then this step can be done with glue-coated slides.

When sectioning is complete, your tissue sections will now be on a slide. We have some sample slides with paraffin sections on them. The sections will store for years in this condition. The next thing that has to be done before you can stain these sections is you have to deparaffinize the sections. If you were to look at the slides with all the paraffin on them, you would never see the tissue for all the paraffin that is there. So the paraffin has to be removed by immersion in xylene or toluene.

Frozen Sections

In frozen section, the piece(s) of tissue to be studied are snap frozen in a cold liquid or cold environment (-20 to -70 °C). Freezing makes the tissue solid enough to section with a microtome.

Frozen sections are performed with an instrument called a cryostat. The cryostat is just a refrigerated box containing a microtome. The temperature inside the cryostat is about -20 to -30 °C. The tissue sections are cut and picked up on a glass slide. The sections are then ready for staining.

General method of Sectioning

1- Place the paraffin wax block into block holder.
2- Put the scale at 20 or more (Microns) which indicates the thickness of the section.
3- Trim and get rid of excess paraffin until reach the surface of the tissue.
4- Put the scale to 7 or 8 microne thickness, and continue cutting or sectioning, until you get a paraffin ribbon.
5- Collect the paraffin ribbon by using a brush and a needle; transfer it to a clean paper.

6-Carry out the knife, clean it with piece of quaze moisture with xylene.

7-All metal parts of microtome must be clean with xylene.

Mounting sections

Mount section by placing them on a pool of dilute albumen solution on a microscope slides, and heated it on a hotplate at about 45° C to expand the sections. The temperature must not exceed the melting point of the paraffin used, usually 45°C. The general method for mounting:

1- Clean the slide with 70% alcohol, to get rid from dust, let dry.
2- Put one drop of adhesive mixture (albumen solution, glycerin) on the slide.
3- With clean finger spread this drop to get a thin film of this mixture and let dry.
4- Put one or two drops of distilled water.
5- Cut the ribbon by using a scalpel and choose one or two sections, transfer it (by the scalpel).
6- Let the slide to stand upright for few seconds.
7- Put the slide on a hotplate regulates slightly below the melting point of the paraffin.
8- Wait for few minutes and collect all slides.
9- Wipe round the sections and leave overnight in an oven at 37 °C to dry completely.

STAINING

The embedding process must be reversed in order to get the paraffin wax out of the tissue and allow water soluble dyes to penetrate the sections. Therefore, before any staining can be done, the slides are

"deparaffinized" by running them through xylene (or substitutes) to alcohols to water. There are no stains that can be done on tissues containing paraffin.

Most cells are essentially transparent, with little or no intrinsic pigment. Even red blood cells, packed with hemoglobin, appear nearly colorless when unstained, unless packed into thick masses. Certain special stains, which bind selectively to particular components, may also be used to identify those structures. But the essential function for staining is simply to make structures easier to see. Staining provides visual contrast and may help identify specific tissue components.

The same structures may have very different colors with different stains. For example, collagen is pink with H&E (hematoxylin and eosin), but blue or green with trichrome. You should generally use specific aspects of actual structure (location, size, shape, texture) to identify cells and tissues, rather than color.

Keep in mind that if the stains are aqueous, you will first have to go back through an alcohol series to rehydrate your tissue before staining can occur. Finally, after staining is complete, it is time to coverslip your slides for viewing. If this is for long-term storage, you will typically have to dehydrate your stained tissue before applying the coverslip.

Staining of the processed histology slides can be done to slides processed by the chemical fixation or frozen section slides. To see the tissue under a microscope, the sections are stained with one or more pigments. The aim of staining is to reveal cellular components; counter stains are used to provide contrast.

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Stains

The list of chemicals used for staining is even longer than that of those used for fixation. Most stains are classified as acids or bases. Actually, they are neutral salts having both acidic and basic radicals. When the coloring property is in the acid radical of the neutral salt, the stain is spoken of as in acid dye, and the tissues which stained with the dye are called acidophilic.

Common acidic components of tissue are nuclei, mucus, cartilage, because these acidic tissue components attract basic dyes.

Method of Staining

Stains can be used in two ways:

1- As direct stains.

In this case the stain and the tissue combine directly, e.g. by an ionic linkage, so that by the end of the staining process the tissue has acquired the color of the dye.

2- As indirect stains.

In this case a third substance, known as a mordant, is required to bind between the tissue and the dye. Mordant is a substance that has a strong affinity for the dye and also for a tissue component. The mordant combines with the tissue and the dye to form a “fast” color combination.

Many fixative solutions contain a mordant, e.g. chromic acid. Many staining solutions contain mordant, e.g. haematoxylin.

A dye sometimes stains electively a single tissue constituent, e.g. resorcin-fuchsin for elastic fibers, this is a differential stain. Most dyes, however, stain all the constituents to varying degrees, this is a diffuse stain.

Counter stains

Use one or more additional stains to show the other components of the tissue. These additional stains called counter stains.

They should be chosen:

1- They must be of a different color.

2- They should be pale to avoid masking the specific stain.

3- The counter staining solution should not itself remove any of the specific stain.

4- Commonly a counter stain is chosen that stains directly, without a mordant.

Hematoxylin &Eosin stain

Hematoxylin and eosin (H&E stain) is the most commonly used light microscopical stain in histology and histopathology (often abbreviated H&E). Other stains are referred to as "special stains" because they are employed in specific situations according to the diagnostic need. Hematoxylin is used to stain nuclei blue, while eosin stains cytoplasm and the extracellular connective tissue matrix pink. Hematoxylin, a basic dye, with deep purple or blue color, stains nuclei blue due to an affinity to nucleic acids in the cell nucleus; eosin, an acidic dye, stains the cytoplasm pink.

Hematoxylin is the oxidized product of the logwood tree known as hematein. Since this tree is very rare nowadays, most hematein is of the synthetic variety. Pure hematoxylin is colorless but it can easily be oxidized to the reddish dye haematin which is active and called heamatoxylin solution. Heamatoxylin solution is only useful after the passage of time (natural oxidizing) which is carried out by exposing the heamatoxylin solution to the air and sunlight. Artificial oxidizing which is carried out by addition of an oxidizing agent.

Hematoxylin will not directly stain tissues, but needs a "mordant" or link to the tissues. This is provided by a metal cation such as iron, aluminum, or tungsten. The variety of hematoxylins available for use is based partially on choice of metal ion used.

Eosin is a red color acidic dye with an affinity for cytoplasmic components of the cell. Structures stained by acid stains are described as acidophilic, and include collagen fibers, red blood cells, muscle filaments, mitochondria. Eosin is much more forgiving than hematoxylin and is less of a problem in the lab. The only problem you will see is overstaining, especially with decalcified tissues.

Note that, basophilic cell structures are NOT necessarily acidic; they only happen to stain with basic stains. Likewise for acidophilic structures, which are NOT necessarily basic. Many tissue staining properties are determined by the complex chemistry of proteins and other macromolecules after interactions with fixatives and other processing agents.

Remember that nuclei are not really purple and collagen is not really pink. If an H&E slide shows any colors other than purple/blue and red/pink -- such as yellow or brown -- the additional color is probably due to an intrinsic pigment such as melanin.

Some cell structures do not stain well with aqueous dyes and so routinely appear clear. This is especially so for those which are hydrophobic, containing fat.

Special Stains in Histology

Hematologic stains

There are a number of special stains employed to identify specific inflammatory cells seen in peripheral blood and tissues. These include Giemsa stains, leukocyte alkaline phosphatase (LAP), tartrate- resistant acid phosphatase (TRAP), and myeloperoxidase (MPO).

Giemsa stain

Giemsa's solution is a mixture of methylene blue, eosin, and azure B. The stain is usually prepared from commercially available Giemsa powder. Giemsa stain is used to visualize chromosomes also stains the fungus histoplasma. Giemsa stain is also a differential stain. It can be used to study the adherence of pathogenic bacteria to human cells. It differentially stains human and bacterial cells purple and pink respectively. It can be used for histopathological diagnosis of malaria and some other protozoan blood parasites.

Giemsa stain is used to differentiate nuclear and/or cytoplasmic morphology of platelets, RBCs, WBCs, and parasites. The most dependable stain for blood parasites, particularly in thick films, is Giemsa stain. Liquid stock is available commercially. The stain must be diluted for use with water buffered to pH 6.8 or 7.0 to 7.2, depending on the specific technique used. Either should be tested for proper staining reaction before use. The stock is stable for years, but it must be protected from moisture because the staining reaction is oxidative. Therefore, the oxygen in water will initiate the reaction and ruin the stock stain. The aqueous working dilution of stain is good only for 1 day.

Specimen

The specimen usually consists of fresh whole blood collected by finger puncture or of whole blood containing EDTA (0.020 g/10 ml of blood) that was collected by venipuncture and is less than 1 h old. Heparin (2 mg/10 ml of blood) or sodium citrate (0.050 g/10 ml of blood) may be used as an anticoagulant if

trypanosome is suspected. If slides have been prepared, the specimen may be a thin blood film that has been fixed in absolute methanol and allowed to dry, a thick blood film that has been allowed to dry thoroughly and is not fixed, or a combination of a fixed thin film and an adequately dried thick film (not fixed). The combination thick/thin blood film is also acceptable.

Quality Control

A. The stock buffer solutions and buffered water should be clear, with no visible contamination.

B. Check the Giemsa stain reagents, including the pH of the buffered water, before each use.

C. Prepare and stain films from "normal" blood, and microscopically evaluate the staining reactions of the RBCs, platelets, and WBCs; this assessment can also be accomplished by the examination of your patient slide. If the staining reactions are acceptable, then the QC is considered acceptable.

a. Macroscopically, blood films appear purplish. If blue, the buffered water was too alkaline; if pink to red, the buffered water was too acid.

b. Microscopically, RBCs appear pinkish gray, platelets appear deep pink, and WBCs have purple-blue nuclei and lighter cytoplasm. Eosinophilic granules are bright purple-red, and neutrophilic granules are purple.

c. Slight variation may appear in the colors described above depending on the batch of stain used and the character of the blood itself.

Procedure

1. Allow the thick film to air dry thoroughly.
2. Fix air-dried thin film in absolute methanol by dipping the film briefly (two dips) in a jar containing absolute methanol. Be sure not to get the alcohol or its fumes on the thick film.
3. Remove and let air dry. Be sure slide is thoroughly dry before staining.

4. Stain the entire slide with diluted Giemsa stain (1:50, vol/vol) for 50 min. For a 1:50 dilution, add 2 ml of stock Giemsa to 40 ml of buffered water in a jar. Place the slide in the stain.
5. Rinse the thin film by briefly dipping the film in a jar of buffered water (one or two dips). Wash the thick film for 3 to 5 min.
6. Let air dry in a vertical position.

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Preparing Chemical Solutions

Lab experiments and types of research often require preparation of chemical solutions in their procedure. We look at preparation of these chemical solutions by weight (w/v) and by volume (v/v).

Glossary, basic terms to understand...

Solute - The substance which dissolves in a solution

Solvent - The substance which dissolves another to form a solution. For example, in a sugar and water solution, water is the solvent; sugar is the solute.

Solution - A mixture of two or more pure substances. In a solution one pure substance is dissolved in another pure substance homogenously. For example, in a sugar and water solution, the solution has the same concentration throughout, it is homogenous.

Introduction to preparation of solutions

Many experiments involving chemicals use in solution form. That is, two or more substances are mixed together in known quantities. This may involve weighting a certain amount of dry material or measuring a certain amount of liquid. Preparing solutions accurately will improve an experiment's safety and chances for success.

Solution 1: Using percentage by weight (w/v)

Formula

> The formula for weight percent (w/v) is: [Mass of solute (g) / Volume of solution (ml)] x 100

Example

A 10% NaCl solution has ten grams of sodium chloride dissolved in 100 ml of solution.

Procedure

Weigh **10g** of sodium chloride. Pour it into a graduated cylinder or volumetric flask containing about **80ml** of water. Once the sodium chloride has dissolved completely, add water to bring the volume up to the final 100 ml. Caution: Do not simply measure**100ml** of water and add 10g of sodium chloride. This will introduce error because adding the solid will change the final volume of the solution and throw off the final percentage.

Solution 2: Using percentage by volume (v/v)

When the solute is a liquid, it is sometimes convenient to express the solution concentration as a volume percent.

Formula

The formula for volume percent (v/v) is: [Volume of solute (ml) / Volume of solution (ml)] x 100

Example

Make 100ml of a 5% by volume solution of ethylene glycol in water.

Procedure

Dissolve **5ml** ethylene glycol in a little less than **95ml** of water. Now bring final volume of solution up to **100ml** with the addition of more water. (This eliminates any error because the final volume of the solution may not equal the calculated sum of the individual components).

Bone Decalcification

Bone is a specialized connective tissue that offers protection to internal organs and works with the musculature to provide physical support and movement. Bone has a hard, rigid nature due to the inclusion of minerals in its osteoid matrix. This crystalline component of the bone, known as hydroxyapatite, serves as a reservoir for a multitude of inorganic ions that are subsequently utilized by various physiological systems. It is composed of calcium, phosphate and hydroxyl ions along with small amounts of magnesium, fluoride, carbonate, citrate, potassium and several other ions.

Bone decalcification is the removal of calcium ions from the bone through histological process thereby making the bone flexible and easy for pathological investigation. This is necessary in order to obtain soft sections of the bone using the microtome. Every thin section cut can be processed like any other soft tissue of the body. Although some labs process mineralized bone for resin embedding, most routine labs demineralize, or "decalcify", bone specimens to make them soft enough to section in paraffin. Bone specimens can be particularly challenging because they are often submitted with the surrounding cartilage and soft tissue that can be easily damaged when exposed to many of the common decalcification solutions.

Specimens submitted for decalcification should be cut into small pieces to expose the marrow, since that is normally the area of interest. Decalcification is carried out after the tissue has been fixed and before it is dehydrated. Decalcification is carried by using decalcifying fluids, the later cause some loss of histological detail and staining qualities, and this loss is greater when decalcification is rapid. Zenker fixative should not be used because it penetrates poorly. 10% formalin is satisfactory although formal saline is used. Temperature is

thought to effect in speed of decalcification and generally temperature below 10 °C slows the process, and best temperature is 40-60 °C.

The selection of the decalcification method and agent should be made after determining their effects on the subsequent diagnostic techniques that will be used on the specimen. There are two commonly used methods of decalcification: acid methods and chelating methods.

Decalcification is a lengthy procedure, as bone pieces have to be left in the decalcifying agent for several days or even weeks, depending on the size of the bone.

There are two categories of decalcifying agents namely, chelating agents and acids. The acids are further divided into weak (picric, acetic and formic acid) and strong acids (nitric and hydrochloric acid). Most frequently used chelating agent is "EDTA". Acids have some effects on the stainability of the tissue.

Acid Method

Calcium salts dissolve and then ionize when exposed to acids. The acids used for decalcification are either inorganic acids (hydrochloric and nitric) or organic acids (formic and acetic). Formic acid is very commonly used because it is fairly slow and gentle to the tissue. Staining is usually very strong, even after prolonged exposure. The inorganic acids remove calcium faster and, when the end point is carefully controlled, are more compatible with immunohistochemistry procedures.

The calcium ions that have been removed can saturate the solution around the specimen and prevent further decalcification if the solution is not agitated and changed regularly. Vacuum will facilitate infiltration and remove carbon dioxide bubbles that form on the specimen surfaces.

Chelating Method

Chelating agents are organic compounds that are capable of binding with certain metals. They are typically very slow acting and gentle, making them good fixatives for electron microscopy and immunohistochemistry studies. Ethylenediaminetetraacetic acid (EDTA) is the most common chelating agent used

for decalcification. It binds with calcium ions and gradually reduces the crystal size of the outer layer of the hydroxyapatite crystal. Care should be taken when specimens contain cartilage because overexposure can remove proteoglycans and weaken staining.

Microtechnique L2 Dr. Muna Al- Rubiae

Preparing Chemical Solutions

Lab experiments and types of research often require preparation of chemical solutions in their procedure. We look at preparation of these chemical solutions by weight (w/v) and by volume (v/v).

Glossary, basic terms to understand...

Solute - The substance which dissolves in a solution

Solvent - The substance which dissolves another to form a solution. For example, in a sugar and water solution, water is the solvent; sugar is the solute.

Solution - A mixture of two or more pure substances. In a solution one pure substance is dissolved in another pure substance homogenously. For example, in a sugar and water solution, the solution has the same concentration throughout, it is homogenous.

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