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TOOLS AND TECHNIQUES IN BIOLOGY

A Laboratory Manual

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A LABORATORY MANUAL

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PREFACE

It is with great pleasure that we present this ***"Tools and Techniques in Biology: A laboratory Manual".****This handbook has been meticulously crafted to serve as a comprehensive guide for students of biological sciences, particularly those pursuing their Master's degree at Sant Gadge Baba Amravati University.*

The field of biological sciences demands not only theoretical knowledge but also practical proficiency in various laboratory techniques. Understanding and mastering these techniques are essential for any aspiring biologist. With this objective in mind, we have compiled a series of practical exercises that cover a wide array of essential methodologies. From the preparation of fixatives and staining techniques to advanced chromatographic separations and spectrophotometric analyses, each practical exercise is designed to enhance practical skills and deepen understanding.

Dr. Mrs. Savita Nalawade and Dr. Mrs. Vijayshree Hemke, whose expertise and dedication have shaped this handbook into a valuable resource. Special thanks to our editor, Dr. Ms. Ashiya Momin, for her meticulous attention to detail and commitment to ensuring the accuracy and relevance of the content.

We extend our gratitude to VYD Publishers, Satara, for their unwavering support in bringing this handbook to completion.

It is our fervent hope that this Practical Handbook will not only serve as a guide but also inspire curiosity and enthusiasm for the fascinating world of biological sciences among our students.

Dr. Mrs. Savita Nalawade **Dr. Mrs. Vijayshree Hemke**

CONTENTS

EXPERIMENT No. 1

PREPARATION OF FIXATIVES

Aim: To prepare fixatives for making tissue sections.

Fixatives are chemical substances used in the preparation of biological tissues for microscopic examination. They work by stabilizing the structure of cells and tissues, preventing degradation and maintaining the morphology and molecular integrity of the sample. Fixatives are essential this by various mechanisms such as protein crosslinking, dehydration and protein denaturation.

Fixatives are crucial for:

1. **Preserving Tissue Morphology:** Maintaining the structure and organization of cells and tissues.
2. **Preventing Autolysis and Putrefaction:** Inhibiting enzymatic degradation and bacterial decay.
3. **Facilitating Staining:** Enhancing the uptake of stains for microscopic examination.
4. **Preserving Molecular Integrity:** Maintaining the structural integrity of proteins, nucleic acids and other cellular components for subsequent analysis.

Fixatives are widely used in:

- **Histology and Pathology:** For routine tissue examination and diagnosis.
- **Cytology:** In the preparation of cell smears and biopsies.

- **Immuno histochemistry**: Preserving antigenicity for antibody based staining.
- **Molecular Biology:** For preserving nucleic acids and proteins for molecular analysis.
- **Electron Microscopy:** Ensuring fine structural preservation of cells and tissues.

1. ALCOCOL FIXATIVE

Alcohols fix tissues by dehydrating cells and denaturing proteins leading to protein precipitation which helps in preserving cellular architecture and nucleic acids.

Materials:

- Ethanol (absolute, 95% or 70%)
- Distilled water
- Measuring cylinder
- Glass container (for mixing)

Procedure:

1. Absolute Ethanol (100%): Use ethanol directly without any dilution.

2. 95% Ethanol: Measure 95 ml of absolute ethanol. Add 5 mL of distilled water. Mix well in a glass container.

3. 70% Ethanol: Measure 70 ml of absolute ethanol. Add 30 mL of distilled water. Mix well in a glass container.

Applications: Used primarily for cytological smears, enzyme histochemistry and preserving tissues for molecular biology studies.

2. ACETONE FIXATIVE

Acetone is a solvent that rapidly dehydrates tissues and precipitates proteins. It also dissolves lipids, making it useful for studying lipidcontaining tissues and enzyme histochemistry.

Materials:

- Acetone
- Distilled water (if dilution is needed)
- Measuring cylinder
- Glass container (for mixing)

Procedure:

1. Pure Acetone: Use acetone directly without any dilution for fixing tissues.

2. 70% Acetone: Measure 70 mL of acetone. Add 30 mL of distilled water.

Mix well in a glass container.

Applications: Frequently used in enzyme histochemistry and immune histochemistry due to its rapid fixation properties and preservation of enzyme activity.

3. FORMALIN (10% Neutral Buffered Formalin)

Formalin, an aqueous solution of formaldehyde fixes tissues by forming crosslinks between lysine residues in proteins, preserving cellular and tissue structure. Buffering the formalin solution helps maintain a neutral pH, minimizing formic acid formation and preventing tissue degradation.

Materials:

- Formaldehyde (37 to 40% solution)

- Distilled water
- Sodium phosphate, monobasic (NaH_2PO_4)
- Sodium phosphate, dibasic (Na_2HPO_4)
- Measuring cylinder
- Balance (for weighing solids)
- Glass container (for mixing)

Procedure:

1. Prepare 10% Formalin: Measure 100 mL of formaldehyde solution.

Dilute to 1 litre with distilled water in a glass container.

2. Prepare Buffer Solution: Weigh 4 g of sodium phosphate, monobasic (NaH_2PO_4). Weigh 6.5 g of sodium phosphate, dibasic (Na_2HPO_4).Dissolve both in the diluted formalin solution.

3. Mixing: Stir the solution until all the salts are fully dissolved.

Applications: Widely used for routine histopathological examination due to its excellent tissue penetration and preservation qualities. It is the most common fixative in diagnostic histology.

4. BOUIN'S FLUID

Bouin's fluid contains picric acid, formaldehyde and acetic acid. Picric acid acts as a precipitating fixative that binds to proteins and nucleic acids. Formaldehyde provides crosslinking of proteins, and acetic acid fixes nucleic acids and helps to preserve cytoplasmic elements. This combination results in excellent preservation of tissue morphology and detail.

Materials:

- Picric acid, saturated aqueous solution

- Formaldehyde (37-40% solution)
- Glacial acetic acid
- Measuring cylinder
- Glass container (for mixing)

Procedure:

1. Mixing Components: Measure 75 mL of saturated picric acid solution. Add 25 mL of formaldehyde solution. Add 5 mL of glacial acetic acid. Mix well in a glass container.

Applications: Commonly used for fixing delicate tissues such as embryos and endocrine tissues, providing good preservation of nuclear and cytoplasmic details.

5. CARNOY'S FLUID

Carnoy's fluid is a mixture of ethanol, chloroform and acetic acid. Ethanol precipitates and fixes proteins by dehydration. Chloroform aids in lipid extraction and acetic acid helps to fix nucleic acids and preserve chromosomal structure. This combination rapidly fixes tissues making it ideal for cytological studies and preserving fine chromosomal details.

Materials:

- Absolute ethanol
- Chloroform
- Glacial acetic acid
- Measuring cylinder
- Glass container (for mixing)

Procedure:

1. Mixing Components: Measure 60 mL of absolute ethanol. Add 30 mL of chloroform. Add 10 mL of glacial acetic acid. Mix well in a glass container.

Applications: Often used for cytological preparations and rapid fixation of small tissue samples, particularly for chromosome studies and biopsies where quick preservation is crucial.

GENERAL SAFETY AND HANDLING TIPS

1. **Personal Protective Equipment (PPE):** Always wear gloves, lab coat, and safety goggles when handling chemicals.
2. **Ventilation:** Work in a well-ventilated area or fume hood to avoid inhaling harmful fumes.
3. **Storage:** Store chemicals in a cool, dry place, away from direct sunlight and heat sources. Ensure containers are properly labelled with contents and hazard warnings.
4. **Disposal:** Dispose of chemical waste according to your institution's safety protocols and local regulations.
5. **Spill Response:** Have spill response materials available, such as absorbent pads and neutralizing agents.

In case of a spill, follow your institution's spill response procedures. By adhering to these detailed procedures and safety guidelines, you can prepare effective fixatives for various histological applications, ensuring the preservation and integrity of tissue samples for accurate examination.

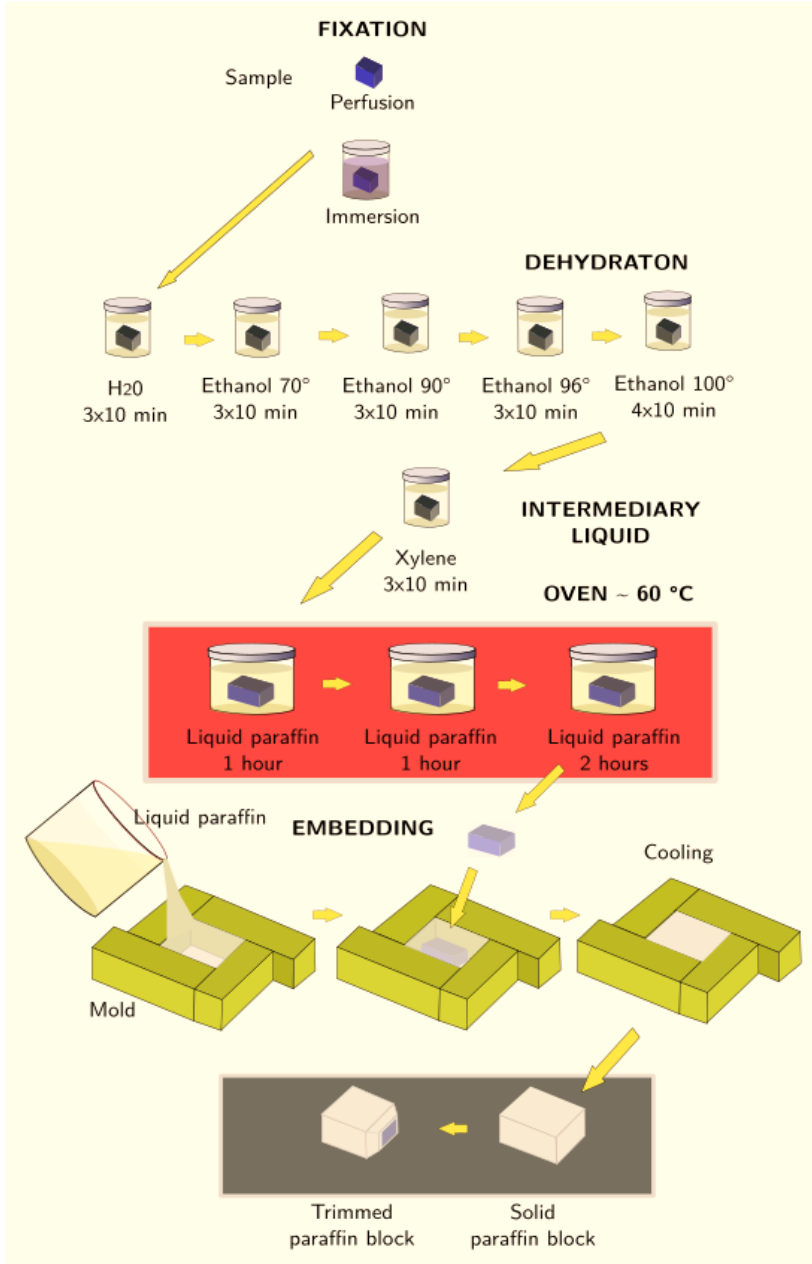


Figure 1: Fixation, Dehydration and Clearing Tissues

EXPERIMENT No. 2

PREPARATION OF ALCOHOLIC GRADES, DEHYDRATION, AND CLEARING OF TISSUES

Aim: To prepare alcoholic grades, dehydration and clearing of tissues.

The preparation of alcoholic grades, dehydration, and clearing of tissues are crucial steps in the histological processing of tissue samples. These steps ensure that tissues are properly prepared for embedding, sectioning and staining, allowing for accurate microscopic examination

ALCOHOLIC GRADES

The preparation of alcoholic grades is important step in the dehydration process of biological tissues for microscopic examination. This method involves creating a series of alcohol solutions with progressively increasing concentrations. By gradually transitioning from lower to higher concentrations of alcohol, tissues are dehydrated in a controlled manner. This gradual approach minimizes the risk of tissue shrinkage or damage ensuring the preservation of cellular structure and integrity for accurate microscopic analysis.

Materials:

- Absolute ethanol (100%)

- 95% ethanol
- 70% ethanol
- 50% ethanol
- Distilled water
- Measuring cylinders
- Glass or plastic containers for each alcohol concentration

Procedure

1. **Absolute Ethanol (100%):** Use ethanol directly from the bottle.
2. **95% Ethanol:** Measure 95 mL of absolute ethanol. Add 5 mL of distilled water. Mix well in a glass container.
3. **70% Ethanol:** Measure 70 mL of absolute ethanol. Add 30 mL of distilled water. Mix well in a glass container.
4. **50% Ethanol:** Measure 50 mL of absolute ethanol. Add 50 mL of distilled water. Mix well in a glass container.

Importance:

Gradual dehydration minimizes tissue shrinkage and distortion. Provides a gentle transition from aqueous to non-aqueous phases.

DEHYDRATION

Dehydration is a crucial step in the preparation of biological tissues for microscopic examination. It involves the systematic removal of water from the tissue samples, which is essential for subsequent embedding in paraffin or resin. This process typically employs a series of alcohol solutions with progressively increasing concentrations. By carefully controlling the dehydration process,

tissues are protected from excessive shrinkage or damage, ensuring that their cellular structures remain intact and suitable for detailed microscopic analysis.

Materials:

- Series of ethanol grades (as prepared above)
- Clearing agents (e.g. xylene, toluene or chloroform)
- Tissue processor or glass containers
- Tissue cassettes
- Timer or clock

Procedure:

1. Start with Lower Concentrations: Place tissue cassettes in 50% ethanol for 1 hour. Transfer to 70% ethanol for 1 hour.
2. Intermediate Concentrations: Move to 95% ethanol for 1 hour. Finally, place in absolute ethanol for 1 hour.
3. Complete Dehydration: Ensure tissues are completely dehydrated by immersing in fresh absolute ethanol for an additional 30 minutes.

Importance:

Essential for embedding in media such as paraffin, which is immiscible with water. Preserves tissue morphology and cellular detail.

CLEARING

Clearing agents replace alcohol in the tissue, making it compatible with the embedding media. Clearing makes tissues transparent and removes the alcohol, replacing it with a medium

that is miscible with both alcohol and embedding media such as xylene or other clearing agents.

Materials:

Series of ethanol solutions (as prepared above)

- Clearing agents (e.g. xylene, toluene or chloroform)
- Tissue processor or glass containers
- Tissue cassettes
- Timer or clock

Procedure:

1. Transfer to Clearing Agent: Place tissue cassettes in xylene or another clearing agent for half to one hour. Repeat in fresh xylene for another hour.
2. Ensure Transparency: Tissues should become translucent indicating successful clearing.

Importance:

Renders tissues transparent allowing for proper infiltration of embedding media. Removes alcohol, facilitating the impregnation with paraffin or other embedding substances.

SAFETY AND HANDLING TIPS

1. **Personal Protective Equipment (PPE):** Always wear gloves, lab coat, and safety goggles when handling alcohol and clearing agents.
2. **Ventilation:** Work in a well-ventilated area or fume hood to avoid inhaling fumes from alcohol and clearing agents.

3. **Storage:** Store alcohol and clearing agents in a cool, dry place away from direct sunlight and heat sources. Ensure containers are properly labelled with contents and hazard warnings.
4. **Disposal:** Dispose of chemical waste according to your institution's safety protocols and local regulations.
5. **Spill Response**: Have spill response materials available, such as absorbent pads and neutralizing agents.

In case of a spill, follow your institution's spill response procedures. By following these detailed procedures and safety guidelines, you can effectively prepare tissue samples for histological examination ensuring the preservation of their structural integrity for accurate analysis.

EXPERIMENT

No. 3

EMBEDDING, BLOCK MAKING AND TRIMMING OF BLOCKS

Embedding is a critical step in histological tissue processing that involves infiltrating tissues with a supportive medium such as paraffin wax which solidifies to form a block. This block can then be sectioned into thin slices for microscopic examination. Block making and trimming ensure that tissues are properly oriented and ready for sectioning.

1. EMBEDDING

Embedding is a vital stage in the preparation of biological tissues for microscopic examination. After dehydration and clearing the tissue samples are infiltrated with a medium such as paraffin wax or resin which provides support and preserves the structural integrity of tissue. This process involves immersing the tissue in the embedding medium which hardens to form a solid block. Proper embedding ensures that thin, precise sections can be cut for microscopic analysis, allowing for detailed observation of cellular and tissue architecture.

Materials

- Paraffin wax
- Embedding molds (metal or plastic)
- Forceps and other tissue handling tools

- Embedding centre or hot plate
- Preheated oven (for paraffin infiltration)

Procedure

1. **Preparation:** Preheat the paraffin wax to the melting point (approximately 56-58°C). Ensure tissues are fully dehydrated and cleared before embedding.
2. **Infiltration:** Place tissues in melted paraffin wax and allow them to infiltrate completely (typically 12 hours in an oven).
3. **Casting:** Pour a small amount of melted paraffin into an embedding mold. Using pre-warmed forceps, carefully orient the tissue in the mold. Fill the mold with more paraffin to cover the tissue completely. Place a labelled tissue cassette on top of the mold to serve as a base once the wax hardens.
4. **Cooling:** Allow the mold to cool and solidify at room temperature, or place it on a cold plate to speed up the process.

Importance and Application: Embedding supports the tissue for thin sectioning. It maintains tissue morphology and integrity. Proper orientation during embedding ensures accurate anatomical representation in sections.

2. BLOCK MAKING

Block making is an essential step in the preparation of biological tissues for microscopic examination. Following the embedding process, tissue samples encased in a solid medium such as paraffin wax or resin are formed into blocks. These blocks provide a stable and uniform matrix enabling precise sectioning of the tissue.

Proper block making is crucial as it ensures that thin slices can be consistently cut and mounted on slides, facilitating detailed microscopic analysis of the tissue's cellular and structural features.

Materials

- Embedding molds
- Tissue cassettes
- Forceps and spatulas

Procedure

1. **Removing the Block:** Once the paraffin has solidified, remove the block from the mold. Trim away any excess paraffin that may obscure the tissue.
2. **Labelling:** Ensure the block is properly labelled with the specimen identification.

Importance and Applications: Block making provides a stable medium for sectioning. It facilitates easy handling and storage of tissue samples. Accurate labelling ensures proper identification and traceability of specimens.

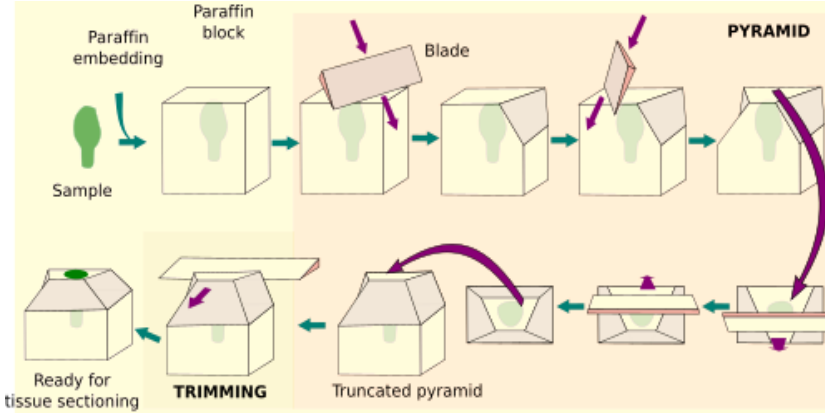


Figure 2: Block Making and Trimming

3. TRIMMING

Trimming is a critical preliminary step in the preparation of embedded tissue samples for microscopic examination. After embedding and block making, the excess embedding medium surrounding the tissue is carefully trimmed away to expose the tissue surface. This process creates a clean and accessible area for precise sectioning. Proper trimming is essential to ensure that the tissue is optimally positioned and oriented, allowing for the production of high-quality, thin sections necessary for detailed microscopic analysis.

Materials

- Microtome
- Blade holder and blades
- Cooling plate or ice tray
- Trimming station or area

Procedure:

1. Preliminary Trimming: Mount the block in the microtome. Use the coarse trimming wheel to trim away excess paraffin until the tissue surface is exposed.

2. Fine Trimming: Adjust the microtome to finer settings. Trim the block to create a flat, smooth surface that will produce uniform sections.

3. Cooling: If necessary, place the trimmed block on a cooling plate to harden the paraffin further before sectioning.

Application: Trimming prepares the block for optimal sectioning. It ensures a flat surface reducing the risk of sectioning artifacts.

Properly trimmed blocks produce high-quality sections for microscopic examination.

SAFETY AND HANDLING TIPS

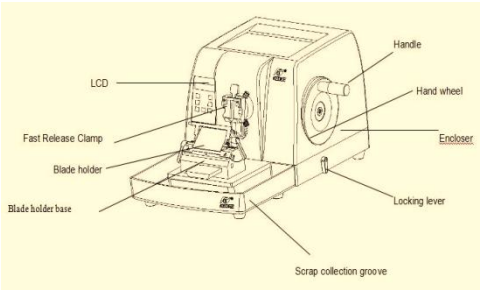
1. **Personal Protective Equipment (PPE):** Wear gloves, lab coat, and safety goggles when handling hot paraffin and working with microtome blades.
2. **Ventilation:** Work in a well-ventilated area to avoid inhaling paraffin fumes.
3. **Heat Safety:** Be cautious when working with heated paraffin to avoid burns.
4. **Blade Safety:** Handle microtome blades with care to prevent cuts. Use a blade holder when changing blades.
5. **Storage:** Store paraffin blocks in a cool, dry place, labelled correctly for easy identification.

By adhering to these detailed procedures and safety guidelines, you can ensure the successful embedding, block making and trimming of tissue samples for histological examination. This process is essential for producing high-quality tissue sections that are crucial for accurate diagnosis and research.

ROTARY MICROTOME

The microtome is a mechanical device for cutting thin, uniform sections of tissue. There are various types of microtome. Rocking microtome, Based sledge, Sliding, Freezing Microtome, Ultra-microtome, Rotary microtome etc. A rotary microtome is common for laboratory practices. Rotary microtome are more handy

in operation and they are available in many models e.g. Spencer type, Erma type with cover etc. Rotary microtome works by rotating the sample across a cutting knife up and down. Typically, a peg holds the samples. The "rotary" term comes from the hand wheel that moves the specimen head up and down. These tissues are obtained for histopathological analysis. The Rotary microtome is a device which maintains the perfect accuracy during sectioning. So it should be handled carefully.



Use of Microtome

1. Bring the feed mechanism backward with return wheel, while doing so disengaged the ratchet wheel, Lock the advanced wheel.
2. Insert the peg on which the trimmed block is mounted into the block clamp so that it will approximate the correct orientation for sectioning. The long edge of the rectangular block should be parallel to the knife edge. Tighten all the screw properly.
3. Insert the honed knife into the knife holder of the microtome. Adjust the angle and clamp screws. Release the advance wheel lock and rotate the wheel. Release the knife

carrier lock and slide the carriage forward until the knife is just in front of paraffin block. Lock the knife carriage in place.

4. Carefully align the block with knife. Lock the advance wheel while aligning the block. The face of the tissue block should be exactly parallel to the knife edge.
5. Release the lock of advance wheel, and move the block towards the knife by turning the advance wheel clockwise. The micron scale should be adjusted at 3-5 μ.
6. Successive Sections should adhere to form a continuous ribbon.
7. After the block is cut completely, remove the knife clean it and return it to its box. Lock the advance wheel and clean the microtome.

EXPERIMENT No. 4

HONING AND STROPPING KNIVES

Honing and stropping are essential steps in maintaining and preparing histological knives for optimal cutting performance. These processes ensure that the knives have a sharp, smooth edge which is critical for producing high-quality tissue sections without damaging the specimen.

HONING

Honing is a meticulous process integral to the refinement of tissue sections prepared for microscopic examination. Following trimming, honing involves delicately refining the surface of the tissue block to achieve optimal section thickness. This step is crucial for producing consistently thin and uniform sections that allow for clear visualization of cellular details under the microscope. By meticulously honing the tissue block, researchers and pathologists ensure the highest quality of microscopic images and accurate interpretation of tissue structures and pathology.

Honing involves the use of a sharpening stone to remove nicks and dull edges from the knife blade. This process re-establishes the blade's edge allowing for precise cuts.

Materials

- Sharpening stone (whetstone or honing stone)

- Honing oil or water (depending on the type of stone)
- Knife holder or guide
- Soft cloth or paper towel

Procedure:

1. **Preparation:** Secure the sharpening stone on a stable surface. If using a whetstone, soak it in water for the recommended time before use. If using an oil stone, apply a thin layer of honing oil.
2. **Angle Setting:** Hold the knife at the correct angle (typically 20-30´degrees) against the stone. Using a knife holder or guide can help maintain a consistent angle.
3. **Honing the Blade:** Place the blade against the stone with the edge facing away from you. Using smooth even strokes, move the blade across the stone and maintaining the angle. Alternate sides to ensure even sharpening. Typically, this involves making a few passes on one side, then switching to the other side. Continue until a burr (a fine wire edge) forms along the entire length of the blade.
4. **Removing the Burr:** Lightly hone the blade with alternating strokes on each side to remove the burr. Wipe the blade with a soft cloth or paper towel to remove any metal filings or oil.

Importance and Applications

Restores the sharp edge of the knife, allowing for clean and precise cuts. Removes nicks and imperfections from the blade, preventing damage to tissue samples during sectioning. Essential for maintaining the longevity and performance of the knife.

STROPPING

Stropping is a crucial technique employed in the preparation of microtome blades used for cutting thin sections of tissue samples in microscopic analysis. This process involves polishing and refining the blade's edge to ensure optimal sharpness and precision. By carefully stropping the blade, any microscopic imperfections and dullness are removed resulting in clean and smooth cuts through tissue samples. This introductory step is essential in achieving high-quality sections that preserve tissue integrity and enable accurate microscopic examination of cellular structures and pathology.

Stropping uses a leather strap or a similar material to polish the blade edge, further refining it and removing any microscopic burrs or roughness left after honing. This step ensures the blade is smooth and exceptionally sharp.

Materials:

- Leather strop
- Stropping compound (optional)
- Knife holder or guide
- Soft cloth or paper towel

Procedure:

1. **Preparation:** Secure the leather strop on a stable surface. If using stropping compound apply a small amount to the strop.
2. **Stropping the Blade:** Hold the knife at the same angle used for honing. Draw the blade across the strop with the edge trailing (opposite direction to honing) to avoid cutting into

the leather. Use smooth, even strokes and apply light pressure. Alternate sides to ensure even polishing.

3. **Polishing the Edge:** Continue stropping until the edge is smooth and polished, free of any burrs. Wipe the blade with a soft cloth or paper towel to remove any residue.

Importance and Applications:

Polishes the blade edge, further refining its sharpness. Removes microscopic burrs left after honing, ensuring a smooth cutting action. Enhances the quality of tissue sections by providing a razor-sharp edge.

SAFETY AND HANDLING TIPS

1. **Personal Protective Equipment (PPE):** Always wear gloves when handling and sharpening knives to prevent cuts. Use safety goggles to protect your eyes from any metal filings or debris.
2. **Stability:** Ensure the sharpening stone and strop are securely placed on a stable surface to prevent slipping during use.
3. **Angle Consistency:** Maintain a consistent angle while honing and stropping to achieve an even, sharp edge.
4. **Pressure Control:** Apply even, moderate pressure during honing and light pressure during stropping to avoid damaging the blade.
5. **Storage:** Store knives in a protective sheath or knife block to maintain their sharpness and prevent accidents.

By following these detailed procedures and safety guidelines, you can ensure that your histological knives are properly honed and

stropped, providing the sharpness necessary for producing high quality tissue sections. This maintenance routine is essential for accurate histological examination and reliable research outcomes.

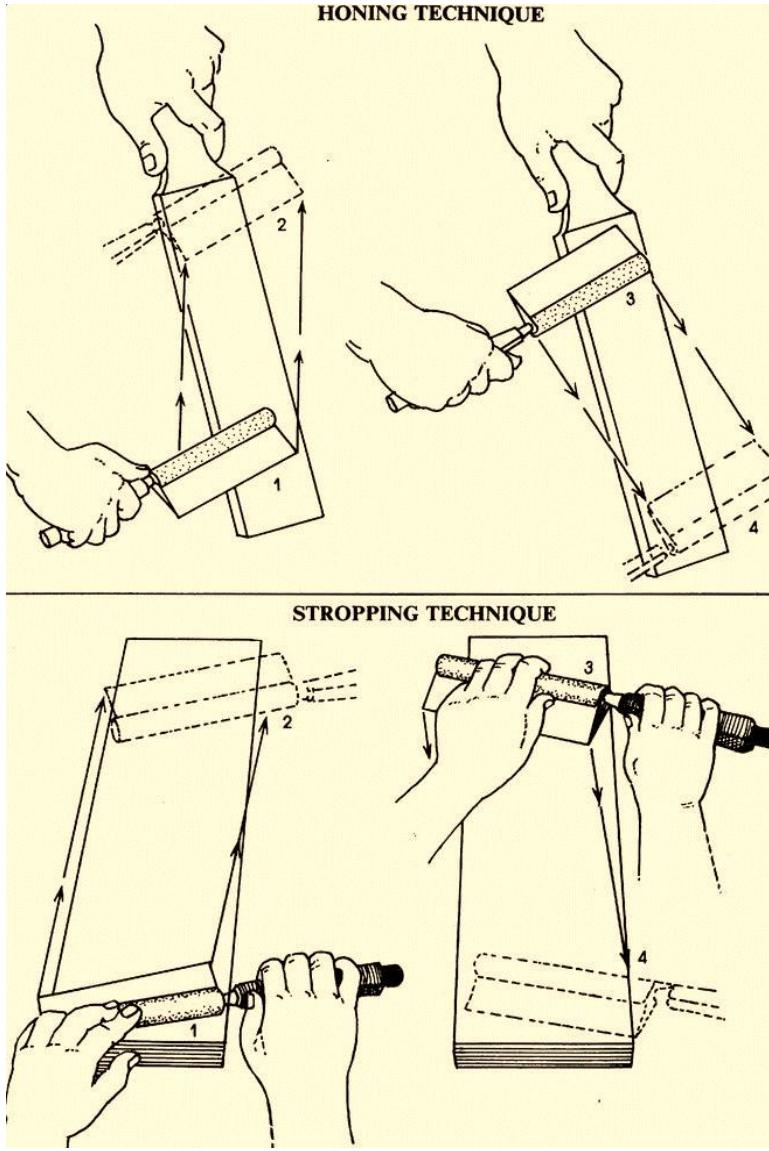


Figure 3: Honing and Stropping Technique

EXPERIMENT

No. 5

SECTION CUTTING AND SPREADING

Section cutting and spreading are critical steps in the preparation of histological slides. After tissues have been fixed, dehydrated, cleared, embedded, and trimmed, thin sections must be cut and spread onto slides for staining and microscopic examination. Proper technique in section cutting and spreading ensures high-quality sections that retain the morphological and structural integrity of the tissue.

SECTION CUTTING

Section cutting is a fundamental procedure in histology and pathology, essential for obtaining thin slices of tissue samples for microscopic examination. Using specialized instruments such as microtomes, tissue blocks embedded in paraffin or resin are sliced into precise sections of uniform thickness. This process requires skill and precision to ensure that sections are thin enough to allow light to pass through for microscopy, yet thick enough to retain tissue structure. Proper section cutting is critical for obtaining clear and detailed images that facilitate the analysis of cellular morphology, tissue architecture, and pathological changes. Section cutting involves using a microtome to slice thin sections of the tissue block.

These sections must be consistent in thickness to ensure uniform staining and clear microscopic visualization.

Materials:

- Microtome
- Sharp microtome blades
- Tissue blocks
- Brushes or forceps
- Water bath (maintained at 40-45°C)
- Clean microscope slides
- Soft tissue paper or blotting paper

Procedure

1. **Preparation:** Ensure the microtome is clean and properly calibrated. Install a new or sharpened blade in the microtome. Place the trimmed tissue block in the block holder of the microtome and secure it tightly.
2. **Setting Thickness:** Set the microtome to cut sections at the desired thickness, typically 3-5 micrometres for routine histology.
3. **Cutting Sections:** Use a smooth, consistent motion to advance the tissue block towards the blade. Carefully slice thin sections, allowing them to float off the blade. Use a brush or forceps to guide the sections from the blade onto a water bath.
4. **Handling Sections:** Float the sections on the surface of the water bath to flatten them. Allow the sections to spread out and eliminate any wrinkles or folds.

Importance and Applications: Produces thin, consistent tissue sections essential for uniform staining and clear microscopic

visualization. Ensures that the morphological and structural integrity of the tissue is preserved.

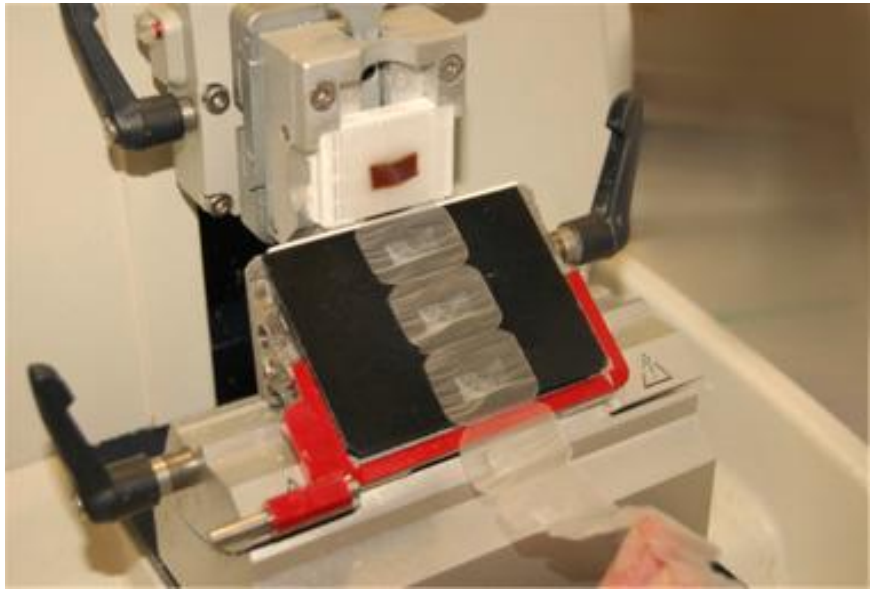


Figure 4: Section Cutting

SPREADING

Spreading is a pivotal step in the preparation of microscopic slides for detailed examination of biological samples. Once tissue sections are cut, they must be carefully spread onto slides to ensure even distribution and proper adherence. This process involves delicately transferring the sections from the cutting instrument to the slide's surface, where they are then flattened and positioned for subsequent staining and analysis. Proper spreading is essential for achieving clear and well-preserved tissue morphology, facilitating

accurate microscopic observation and analysis of cellular structures and pathological features.

Spreading involves placing the thin sections on a microscope slide, ensuring they are flat and free of wrinkles or folds. Proper spreading is critical for the adherence of the sections to the slide and for subsequent staining.

Materials:

- Water bath
- Clean microscope slides
- Fine brushes or forceps
- Slide drying rack
- Adhesive (optional, such as egg albumin or commercial adhesives)

Procedure:

1. **Preparation of Slides:** Clean the microscope slides thoroughly to remove any grease or dust. Optionally, coat the slides with an adhesive to enhance section adherence.
2. **Transferring Sections:** Using fine brushes or forceps, gently pick up the sections from the water bath. Place the sections flat onto the clean microscope slides. Ensure that the sections are spread out evenly without any folds or wrinkles.
3. **Drying the Slides:** Place the slides on a slide drying rack. Allow the slides to air dry or place them in a slide dryer at 37-40°C until they are completely dry.
4. **Final Check:** Examine the slides under a low power microscope to ensure that the sections are flat and free of artifacts.

Importance and Applications: Ensures that sections are flat and well adhered to the slide, preventing artifacts during staining. Facilitates even staining and accurate microscopic examination.

Figure 5: Spreading of Sections on Hot Plate

SAFETY AND HANDLING TIPS

1. **Personal Protective Equipment (PPE):** Always wear gloves, a lab coat, and safety goggles when working with microtome blades to prevent cuts and protect against potential hazards.
2. **Microtome Safety:** Handle microtome blades with care. Use blade holders and avoid touching the cutting edge. Ensure the microtome is properly secured and operated according to the manufacturer's instructions.

3. **Water Bath Safety:** Maintain the water bath at an appropriate temperature (40-45°C) to prevent overheating and ensure optimal section spreading. Use caution when handling hot water to avoid burns.
4. **Slide Handling:** Handle slides by the edges to avoid fingerprints and contamination. Store prepared slides in a dust free environment to maintain section quality.

By adhering to these detailed procedures and safety guidelines, you can ensure the successful cutting and spreading of tissue sections for histological examination. These steps are critical for producing high-quality slides that provide accurate and reliable results in histological analysis.

EXPERIMENT No. 6

PREPARATION OF STAINS: BORAX CARMINE, ACETOCARMINE, ACETOORCEIN AND HAEMATOXYLIN AND EOSIN STAIN

The preparation of stains is a critical component of histological and biological research, essential for enhancing the contrast and visibility of cellular and tissue structures under the microscope. Stains are chemical substances that selectively colour specific components of cells or tissues, highlighting their morphology and aiding in the identification of cellular features and abnormalities. This process involves the meticulous preparation of staining solutions, ensuring their proper concentration and compatibility with the biological specimen being studied. Effective staining techniques are pivotal in enabling researchers and pathologists to accurately interpret and analyse microscopic images, contributing to advancements in medical diagnostics and biological sciences. Different stains highlight various components of cells and tissues, providing insights into their morphology and function.

BORAX CARMINE STAIN

The preparation of Borax Carmine stain is a classical technique in histology, valued for its ability to selectively stain cell nuclei, aiding in the detailed microscopic examination of tissue samples. This stain is prepared using carmine dye dissolved in a borax solution, which acts as a mordant to enhance the staining affinity for nuclear material. The process involves precise

measurements and mixing to achieve the optimal concentration of stain, ensuring consistent and reliable results. Borax Carmine stain is particularly useful in highlighting nuclei in various tissues, providing crucial insights into cellular structure and pathology. Its application remains integral in both research and diagnostic laboratories, supporting the comprehensive study of biological specimens under the microscope.

Borax Carmine Stain Preparation

Materials:

- Carmine (1 g)
- Borax (4 g)
- Distilled water (100 mL)
- Ethanol (70%)

Procedure:

1. Dissolve 4g of borax in 100 mL of distilled water.
2. Add 1g of carmine to the borax solution.
3. Heat the mixture gently in a water bath until the carmine is completely dissolved (do not boil).
4. Allow the solution to cool to room temperature.
5. Filter the solution through filter paper to remove any undissolved particles.
6. Transfer the filtered solution to a dark bottle and store at room temperature.

Applications:

- **Histology:** Used to stain whole mounts and tissue sections, providing clear differentiation of cellular structures.

- **Cytology:** Useful for staining chromosomes during cell division, making it valuable in karyotyping and the study of mitosis and meiosis.
- **Botanical Studies:** Applied in the examination of plant tissues, particularly for highlighting lignified and suberized tissues.

ACETOCARMINE STAIN

The preparation of Acetocarmine stain is a fundamental technique in histology and cytology, widely used for staining chromosomes and other cellular structures for microscopic examination. This stain is made by dissolving carmine dye in acetic acid, which acts as both a solvent and a mordant to enhance the staining specificity and intensity. Acetocarmine stain is particularly valued for its ability to vividly colour chromosomes, facilitating the study of their number, structure, and behaviour during cell division.

The process of preparing Acetocarmine stain involves careful measurement and mixing to achieve the desired concentration and pH level, ensuring optimal staining results. This stain is indispensable in various research fields, including genetics, developmental biology, and pathology, where precise visualization of cellular components is essential for understanding cellular processes and identifying abnormalities. Its versatility and effectiveness make Acetocarmine stain a cornerstone in microscopic analysis, supporting the advancement of biological sciences and medical diagnostics.

Acetocarmine Stain Preparation

Materials:

- Carmine (1 g)
- Glacial acetic acid (45 mL)
- Distilled water (55 mL)

Procedure:

1. Mix 45 mL of glacial acetic acid with 55 mL of distilled water.
2. Add 1 g of carmine to the acetic acid solution.
3. Heat the mixture gently in a water bath until the carmine is completely dissolved (do not boil).
4. Allow the solution to cool to room temperature.
5. Filter the solution through filter paper to remove any un dissolved particles.
6. Transfer the filtered solution to a dark bottle and store at room temperature.

Applications:

1. **Cytogenetics:** Widely used for staining chromosomes in root tip squashes, meristematic tissues, and meiotic cells.
2. **Chromosome Studies:** Essential for identifying chromosomal abnormalities and studying karyotypes. Also used in plant breeding and genetics to observe chromosome behaviour during cell division.

ACETO-ORCEIN STAIN

The preparation of Aceto-Orcein stain is a crucial technique in cytology and histology, specifically used for staining chromosomes and cellular structures to facilitate detailed

microscopic examination. This stain is formulated by dissolving orcein dye in acetic acid, which serves as both a solvent and a mordant to enhance the stain's affinity for chromatin and other cellular components. Aceto-Orcein stain is highly valued for its ability to provide clear and distinct staining of chromosomes, aiding in the visualization and analysis of chromosomal structure, number, and behaviour during cell division.

The process of preparing Aceto-Orcein stain involves precise measurements and mixing to achieve the optimal concentration and pH, ensuring consistent and reliable staining results. This stain is essential in genetic research, cytogenetics, and diagnostic pathology, where accurate visualization of chromosomal abnormalities is critical for understanding genetic disorders and developmental processes. Its application underscores its importance in advancing scientific knowledge and medical diagnostics through the detailed examination of cellular and genetic material under the microscope.

Materials:

- Orcein (1 g)
- Glacial acetic acid (45 mL)
- Distilled water (55 mL)

Procedure:

1. Mix 45 mL of glacial acetic acid with 55 mL of distilled water.
2. Add 1 g of orcein to the acetic acid solution.
3. Heat the mixture gently in a water bath until the orcein is completely dissolved (do not boil).
4. Allow the solution to cool to room temperature.

5. Filter the solution through filter paper to remove any un dissolved particles.
6. Transfer the filtered solution to a dark bottle and store at room temperature.

Applications:

1. **Cytogenetics:** Used to stain chromosomes in both animal and plant cells, making it a key tool for studying mitosis and meiosis.
2. **Genetics Research:** Facilitates the examination of chromosomal structure and behaviour. Commonly used in teaching laboratories for demonstrating chromosome morphology.

HEMATOXYLIN AND EOSIN (H&E) STAIN

The preparation of Hematoxylin and Eosin (H&E) stain represents a cornerstone in histological staining techniques widely regarded for its versatility and ability to highlight different tissue components for microscopic examination. H&E stain consists of two main components: hematoxylin, a natural dye extracted from the heartwood of certain trees, and eosin, a synthetic dye.

Hematoxylin stains nuclei and other basophilic structures (those that have an affinity for basic dyes), appearing blue-purple under the microscope. In contrast, eosin stains cytoplasm and other acidophilic structures (those that have an affinity for acidic dyes), appearing pink or red. This dual staining method provides excellent contrast and allows for the differentiation and visualization of cellular morphology, tissue architecture, and pathological changes.

The process of preparing H&E stain involves several steps, including the preparation of hematoxylin and eosin solutions, their sequential application to tissue sections, and the subsequent dehydration and mounting of stained slides. H&E staining is essential in routine histopathology, enabling pathologists to examine tissue samples to diagnose diseases, assess tissue health, and study normal and abnormal cellular structures. Its widespread use in research and clinical settings underscores its significance in advancing our understanding of biological processes and diseases at the microscopic level.

Hematoxylin and eosin (H&E) staining is one of the most widely used techniques in histology and pathology. Hematoxylin stains cell nuclei blue, while eosin stains the cytoplasm and extracellular matrix pink.

Hematoxylin Preparation

Materials:

- Hematoxylin (1 g)
- Ethanol (absolute, 10 mL)
- Ammonium or potassium alum (50 g)
- Distilled water (1000 mL)
- Sodium iodate (0.2 g)
- Glacial acetic acid (20 mL)
- Glycerol (50 mL)

Procedure:

1. Dissolve 1 g of hematoxylin in 10 mL of absolute ethanol.
2. Dissolve 50 g of ammonium or potassium alum in 1000 mL of distilled water.

3. Add the hematoxylin solution to the alum solution.
4. Add 0.2 g of sodium iodate to oxidize the hematoxylin solution.
5. Add 20 mL of glacial acetic acid and 50 mL of glycerol to the solution.
6. Allow the solution to ripen by storing it in the dark for at least a month (naturally ripened) or by using a chemical oxidizer for immediate use.
7. Filter the solution before use.

Eosin Preparation

Materials:

- Eosin Y (1 g)
- Distilled water (100 mL)
- Ethanol (95%, 100 mL)

Procedure:

1. Dissolve 1 g of eosin Y in 100 mL of distilled water.
2. Add 100 mL of 95% ethanol to the eosin solution.
3. Filter the solution through filter paper.
4. Transfer the filtered solution to a dark bottle and store at room temperature.

Applications:

1. **Histopathology:** The primary stain used in diagnostic pathology for examining tissue biopsies, identifying normal and pathological tissue structures.
2. **Medical Diagnosis:** Essential in the diagnosis of diseases, including cancer, by revealing morphological details.

3. **Research:** Used in a variety of research settings to study tissue architecture and cellular morphology.

General Staining Procedure Using H&E

Materials:

- Slides with tissue sections
- Hematoxylin stain
- Eosin stain
- Distilled water
- 95% Ethanol
- Xylene
- Mounting medium

Procedure:

1. **Deparaffinise and Hydrate:** Deparaffinise tissue sections by placing slides in xylene, followed by hydration through graded alcohols (100%, 95%, 70%) to distilled water.
2. **Stain with Hematoxylin:** Stain sections in hematoxylin for 5-10 minutes.
3. **Rinse:** Rinse slides in running tap water for 5 minutes.
4. **Differentiate:** Differentiate in 1% acid alcohol (1% HCl in 70% ethanol) for a few seconds.
5. **Bluing:** Place slides in a bluing solution (e.g., 0.2% ammonia water or Scott's tap water) for 23 minutes.
6. **Rinse:** Rinse slides in running tap water.
7. **Stain with Eosin:** Stain sections in eosin for 12 minutes.
8. **Dehydrate:** Dehydrate sections through graded alcohols (70%, 95%, 100 %).
9. **Clear:** Clear sections in xylene.

10. **Mount:** Mount sections with a suitable mounting medium.

These stains and their respective procedures are essential tools in biological and medical research, enabling detailed visualization of cellular and tissue structures for diagnostic and educational purposes.

EXPERIMENT

No. 7

STAINING OF SECTIONS, DOUBLE STAINING AND MOUNTING

STAINING

Staining of sections is a fundamental process in histology and pathology, essential for enhancing the visibility and contrast of cellular and tissue structures under the microscope. This technique involves applying specific dyes or stains to tissue sections mounted on slides, selectively Colouring different cellular components based on their chemical properties and affinity for the stain. Staining of sections serves multiple purposes, including highlighting nuclei, cytoplasmic details, connective tissues, and pathological changes, thereby enabling researchers and pathologists to analyze cellular morphology, tissue architecture, and disease processes in detail.

The choice of stain depends on the research or diagnostic objectives, with various stains offering different Colours and specificity for specific cellular structures or pathological features. Proper staining techniques are critical to achieving consistent and reliable results, ensuring that stained sections accurately reflect the characteristics of the tissue under examination. Staining of sections is a foundational step in microscopic analysis, playing a crucial role in advancing biomedical research, clinical diagnostics, and the understanding of physiological and pathological conditions at the cellular level.

Materials:

- Tissue sections on slides
- Stains (e.g., Haematoxylin, Eosin, etc.)
- Distilled water
- Graded alcohol series (70%, 95%, 100%)
- Xylene
- Staining reagents (acid alcohol, bluing solution)
- Mounting medium

Procedure:

1. Deparaffinization and Hydration:

- Place slides in xylene to remove paraffin (3 changes, 5 minutes each).
- Hydrate sections through graded alcohol series (100%, 95%, 70%) to distilled water.

2. Staining:

Haematoxylin Staining:

- Stain sections in haematoxylin for 5 to 10 minutes.
- Rinse in running tap water for 5 minutes.
- Differentiate in 1% acid alcohol (1% HCl in 70% ethanol) for a few seconds.
- Rinse briefly in tap water.
- Blue in a bluing solution (e.g., 0.2% ammonia water) for 23 minutes.
- Rinse in tap water.

Eosin Staining:

- Stain sections in eosin for 12 minutes. Rinse in distilled water to remove excess eosin.

3. Dehydration: Dehydrate sections through graded alcohol series (70%, 95%, 100%).

4. Clearing: Clear sections in xylene (3 changes, 5 minutes each).

5. Mounting: Place a drop of mounting medium on the section. Cover with a cover slip, avoiding air bubbles. Allow to dry.

Applications

- **Histopathology:** Diagnosing diseases by examining stained tissue sections under a microscope.
- **Research:** Studying tissue morphology, cellular structures, and biological processes. Teaching students about tissue architecture and histological techniques.

DOUBLE STAINING

Double staining is an advanced technique in histology and microscopy that involves using two different stains to simultaneously highlight distinct cellular components or structures within tissue samples. This method allows researchers and pathologists to observe multiple features of interest within the same specimen, providing valuable insights into cellular interactions, tissue architecture, and pathological changes.

The process of double staining typically involves sequential application of two compatible stains, each targeting specific components based on their chemical properties and affinity. By carefully selecting stains with contrasting colours or properties, such as hematoxylin and eosin (H&E), researchers can differentiate between nuclei, cytoplasm, collagen fibres, or other cellular elements within a single tissue section.

Double staining enhances the visual contrast and specificity of microscopic images, enabling more comprehensive analysis and interpretation of tissue morphology and pathology. This technique is widely used in research settings to investigate complex biological structures and interactions, offering detailed insights that single stains may not provide. Its application in diagnostic pathology also aids in precise identification and characterization of diseases, contributing to advancements in both basic science and clinical practice.

Materials:

- Tissue sections on slides
- Primary stain (e.g., Haematoxylin)
- Secondary stain (e.g., Eosin)
- Distilled water
- Graded alcohol series (70%, 95%, 100%)
- Xylene
- Mounting medium

Procedure:

1. **Deparaffinization and Hydration:** Follow the same deparaffinization and hydration steps as for single staining.
2. **Primary Staining:** Stain sections with the primary stain (e.g., Haematoxylin) following the single staining procedure.
3. **Differentiation and Bluing:** Differentiate and blue as described in the single staining procedure.
4. **Secondary Staining:** After rinsing, stain sections with the secondary stain (e.g., Eosin) for 12 minutes. Rinse in distilled water to remove excess stain.

5. **Dehydration:** Dehydrate sections through graded alcohol series (70%, 95%, 100%).
6. **Clearing:** Clear sections in xylene (3 changes, 5 minutes each).
7. **Mounting:** Mount the sections as described in the single staining procedure.

Applications:

- **Differentiation:** Highlighting different components of tissues, such as connective tissue and nuclei.
- **Pathology:** Identifying multiple features of a specimen simultaneously, aiding in more accurate diagnoses.
- **Developmental Biology:** Studying the interaction between different tissue types during development.

MOUNTING

Mounting is a crucial final step in the preparation of microscopic slides, ensuring the preservation, protection, and presentation of stained tissue sections for detailed examination. This process involves carefully applying a mounting medium, typically a clear resinous substance or aqueous mounting media, over the stained tissue section on a microscope slide. The goal of mounting is to secure the tissue section in place, prevent dehydration, and create a transparent cover that allows light to pass through for microscopy.

Proper mounting is essential for maintaining the integrity and longevity of stained slides, ensuring that the tissue sections remain stable and undisturbed during examination. It also enhances the clarity and visibility of cellular structures and details, facilitating

accurate observation and analysis under the microscope. Mounting is a critical step in both research and diagnostic histology, providing researchers, pathologists, and clinicians with the ability to study and diagnose diseases, assess tissue health, and contribute to advancements in biomedical sciences and patient care.

Materials:

- Stained tissue sections on slides
- Mounting medium (e.g. Canada balsam, DPX)
- Cover slips
- Forceps
- Drying oven (optional)

Procedure:

1. **Clearing:** Ensure sections are fully cleared in xylene before mounting.
2. **Applying Mounting Medium:** Place a small drop of mounting medium on the tissue section.
3. **Cover slip Application:** Gently lower a coverslip onto the mounting medium using forceps. Avoid trapping air bubbles under the coverslip.
4. **Drying:** Allow the mounted slides to dry in a flat position. Place slides in a drying oven if a faster drying process is required.
5. **Labelling and Storage:** Label the slides with relevant information (e.g., specimen ID, date). Store the slides in a slide box for future examination and reference.

Applications:

- **Preservation:** Protecting stained sections from damage and degradation.
- **Examination:** Providing a stable and clear medium for viewing stained sections under a microscope.
- **Archiving:** Creating permanent records of specimens for future reference.

The staining of sections, double staining and mounting are integral techniques in histology and microscopy. They provide detailed visualization of cellular and tissue structures, facilitating diagnosis, research, and education. The procedures outlined ensure high-quality preparation of specimens, preserving them for accurate and thorough microscopic examination.

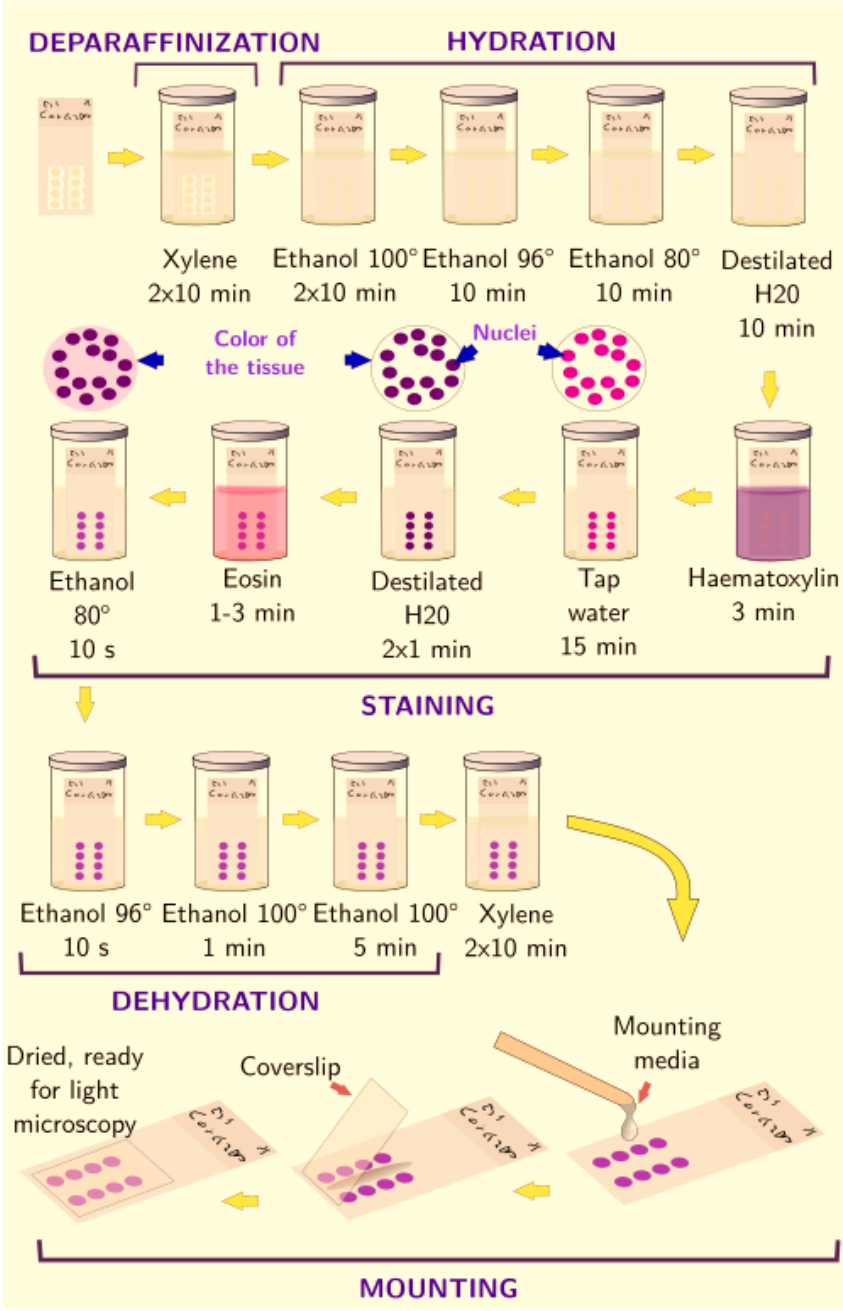


Figure 6: Staining and Mounting

EXPERIMENT No. 8

DETERMINATION OF ABSORPTION SPECTRUM OF HAEMOGLOBIN

Aim: To determine the absorption spectrum of hemoglobin using a spectro-photometer.

Principle:

Absorption spectroscopy, based on Lambert-Beer's law, correlates the absorption of light by a substance to its concentration and the path length of light through the sample. Hemoglobin absorbs light in the visible range due to its heme component.

Materials:

- EDTA (Ethylene diamine tetraacetic acid)
- Whole blood sample
- Distilled water
- Spectrophotometer
- Cuvettes (2)
- Graph paper
- White paper strip
- Notebook for recording data
- Reference materials for wavelength Colours

Procedure:

1. **Prepare and label two cuvettes:**
   - **Cuvette A:** 4 ml of distilled water

- **Cuvette B:** 4 ml of oxyhemoglobin solution prepared from the blood sample with 2 drops of EDTA diluted in distilled water.

2. Set up the spectrophotometer:

- Place Cuvette A in the spectrophotometer as the reference cuvette and zero the absorbance reading.
- Measure the absorbance of Cuvette B from 400 nm to 700 nm at intervals of 10 nm, recording absorbance readings where noticeable changes occur and every 5 nm where absorbance changes are subtle.

3. Observe the Colour spectrum:

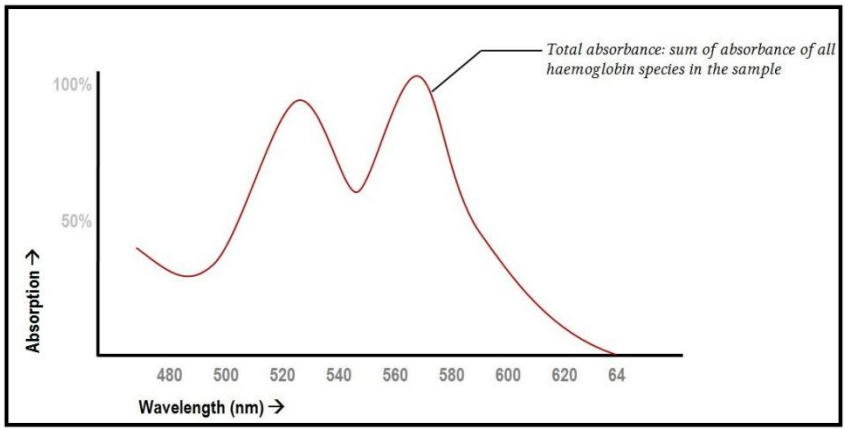
- Place a white paper strip in a cuvette and insert it into the sample holder.
- With the cover open, slowly adjust the wavelength control from 650 nm to 400 nm, noting the wavelengths where the incident light changes Colour from dark red to subtle violet.

4. Record data:

- Record absorbance readings (Y-axis) versus wavelength (X-axis) on graph paper.
- Note the observed Colours corresponding to each wavelength range observed during the spectrum scan.

Observations:

1. The absorption spectrum of oxyhemoglobin shows distinct peaks and valleys across the visible spectrum.
2. Colours observed range from dark red (~650 nm) to subtle violet (~400 nm) as the wavelength decreases.



Results:

- A graph plotting absorbance versus wavelength reveals the absorption spectrum of hemoglobin.
- Peaks in the spectrum indicate wavelengths where hemoglobin absorbs light most strongly, corresponding to its heme structure.

EXPERIMENT No. 9

SPECTROPHOTOMETRIC DETERMINATION OF DYES

Aim: To determine the concentration of red dye in a solution using spectro-photometric methods.

Principle:

Spectroscopy involves measuring a substance's interaction with electromagnetic radiation. This technique can be used to determine the concentration of dyes in solutions by measuring absorbance at specific wavelengths. The absorbance of a solution is directly proportional to the concentration of the absorbing species, as described by Beer's Law: $A=\varepsilon \cdot l \cdot c$, where A is absorbance, ε\varepsilon is molar absorptivity, l is the path length, and c is concentration.

Materials:

- Beakers (50 ml, 100 ml)
- Watch glass
- Graduated pipettes (1 ml, 5 ml) and bulb
- Funnel
- Transfer pipette
- Volumetric flasks (10 ml, 100 ml) with stoppers
- UV is spectrophotometer
- Two cuvettes
- Distilled water

- Red dye stock solution (1.36 x 10^4 M)
- Dropper
- Soft tissue for cleaning cuvettes
- Graph paper or software for plotting data

Procedure:

Part 1: Preparation of Standard Solutions

1. Transfer 25 ml of the stock red dye solution into a small, dry beaker.
2. Using a volumetric pipette, transfer 10 ml of the stock solution to a 100 ml volumetric flask. Add distilled water up to the fill line. Stopper the flask and invert it at least five times to mix the solution. Label this solution A. Calculate the concentration of Solution A.
3. Pipette out 7 ml of the stock solution into a 100 ml volumetric flask. Add distilled water up to the fill line. Stopper the flask and invert it at least five times to mix the solution. Label this solution B. Calculate the concentration of Solution B.
4. Pipette 5 ml of the stock solution into a 100 ml volumetric flask. Add distilled water up to the fill line. Stopper the flask and invert it at least five times to mix the solution. Label this solution C. Calculate the concentration of Solution C.
5. Pipette out 2 ml of the stock solution into a 100 ml volumetric flask. Add distilled water up to the fill line. Stopper the flask and invert it at least five times to mix the solution. Label this solution D. Calculate the concentration of Solution D.
6. Pre rinses each cuvette with the corresponding solution and transfer each prepared standard solution into different

cuvettes. Fill each cuvette about two thirds full. Place the cuvettes into a rack.

7. Fill one cuvette with distilled water after pre rinsing it. This will be used as the blank.
8. Estimate the Colour intensity of the unknown solution by comparing it to the standards against a white background. Record your estimate.
9. Wipe the outside of each cuvette with a soft tissue to remove any liquid or fingerprints.

Part 2: Absorbance Measurements

1. Set the spectrophotometer to the wavelength corresponding to the maximum absorbance (λmax) for the red dye.
2. Set the spectrophotometer to %Transmittance mode. Adjust to 0% T with an empty sample chamber.
3. Insert the blank cuvette filled with distilled water, close the cover and adjust to 100% T.
4. Remove the blank and ensure the spectrophotometer reads 0% T when empty. Recalibrate if necessary.
5. Switch to Absorbance mode. The display will show the absorbance reading for the sample.
6. Measure and record the absorbance of each standard solution and the unknown solution.
7. Verify data consistency. Ensure that absorbance increases with concentration and that the unknown's absorbance falls within the standards range.

8. Clean and store all glassware properly. Dispose of dye solutions by flushing with water. Rinse cuvettes thoroughly. Turn off the spectrophotometer.

Data and Analysis

λ used = _____

Solutions	Concentration (M)	Absorbance at λ
Standard A		
Standard B		
Standard C		
Standard D		
Unknown		

1. Construct a Beer's Law plot of Absorbance (y-axis) vs Concentration (x-axis) using the four standards. Label the axes appropriately. This plot is also known as a Calibration Curve.
2. Draw a trend line through the data points. Include the equation of the line and the R^2 value on the plot.
3. Equation of the line: _____
4. If the path length (L) of the cuvette is 1.00 cm, determine the molar absorptivity (ε) for the red dye at λ max. Report the value with units.
5. ε\varepsilon = _____

6. Using the absorbance of the unknown solution and the equation of the trend line, calculate the concentration of the unknown. Show your work.
7. Explain the purpose of a calibration curve in quantitative analysis.

EXPERIMENT No. 10

SEPARATION OF EMULSION BY SIMPLE CENTRIFUGATION

Centrifugation is a fundamental technique in laboratories for separating emulsions and other mixtures based on density differences, and this experiment highlights its application in separating immiscible liquid components effectively.

Aim: To separate the components of an emulsion using the technique of simple centrifugation.

Principle:

Centrifugation is a process that uses centrifugal force to separate particles or droplets of different densities suspended in a liquid. An emulsion is a mixture of two immiscible liquids where one liquid is dispersed as small droplets within the other. When subjected to centrifugation, the denser liquid droplets will move outward and settle at the bottom, while the less dense liquid will form a layer on top. This separation occurs because the centrifugal force accelerates the sedimentation process based on density differences.

Requirements:

1. Emulsion sample: An example could be an oil in water or water in oil emulsion.
2. Centrifuge: Capable of maintaining controlled speeds (e.g., up to 3000 rpm).

3. Centrifuge tubes: Clean and dry, capable of withstanding centrifugal force.
4. Pipettes: For transferring the emulsion into centrifuge tubes.
5. Timer or stopwatch: To measure the centrifugation time accurately.
6. Marker: For marking levels of separated components.
7. Balance: To measure the volume of emulsion used accurately.
8. Gloves and safety goggles: For safe handling of chemicals and equipment.

Procedure:

1. Preparation of Emulsion Sample:

a. Ensure the emulsion is well mixed before starting the experiment.
b. Use a pipette to transfer a measured volume (e.g., 5 ml) of the emulsion into clean centrifuge tubes.

2. Setting up the Centrifuge:

a. Balance the centrifuge by placing tubes with equal volumes of emulsion (or water if a single sample) in opposite holders to maintain balance.
b. Secure the centrifuge lid to prevent accidents during operation.

3. Centrifugation:

a. Set the centrifuge to a suitable speed (e.g., 2000-3000 rpm). The exact speed may vary based on the density difference between the two liquids in the emulsion.

b. Set the timer for a specific duration (e.g., 10-15 minutes). This time may need to be adjusted based on the emulsion's characteristics.
c. Start the centrifuge and allow it to run for the set duration.

4. Observation:

- After the centrifugation is complete, carefully remove the centrifuge tubes without disturbing the separated layers.
- Observe the tubes and note the distinct layers formed. Typically, the denser liquid will be at the bottom and the less dense liquid will be at the top.
- Use a marker to mark the levels of the separated components for measurement.

5. Measurement and Analysis:

- Measure the volume of each separated layer using a pipette or graduated cylinder.
- Note the clarity and appearance of each layer to assess the effectiveness of the separation.

Results:

- The results should show distinct layers of separated liquids in the centrifuge tube.
- The volume and appearance of each layer can be recorded to determine the efficiency of the separation process.
- The separation efficiency can be analyzed based on the clarity of each layer and the complete segregation of the two immiscible liquids.

EXPERIMENT

No. 11

SEDIMENTATION OF RED BLOOD CELLS BY CENTRIFUGATION

Aim: To determine the sedimentation rate of red blood cells (erythrocytes) using the Westergren method under the influence of centrifugal force.

The Westergren method is a well-established and widely used technique for measuring Erythrocyte Sedimentation Rate (ESR) and is commonly used in clinical and research settings to assess various health conditions.

Principle:

Centrifugation is a technique that uses centrifugal force to separate substances of different densities in a solution. In the case of red blood cells, when a sample of blood is subjected to centrifugation, the denser components such as red blood cells, will settle to the bottom of the test tube due to the gravitational force exerted during centrifugation. The rate at which the red blood cells sediment depends on factors such as cell size, shape and plasma viscosity.

Requirements:

1. Blood sample: Typically anti-coagulated whole blood (e.g., EDTA treated blood).
2. Centrifuge: Capable of maintaining controlled speeds (e.g., up to 2000 rpm).

3. Westergren tubes: These are special tubes used specifically for measuring erythrocyte sedimentation rate (ESR).
4. Westergren stand: To hold the tubes vertically during the measurement.
5. Timer or stopwatch: To measure the sedimentation time accurately.
6. Marker: For marking the initial and final sedimentation levels on the test tube.
7. Balance: To measure the volume of blood used accurately.

Procedure:

1. Preparation of Blood Sample:

a. Collect a small volume (usually 12 ml) of anti-coagulated whole blood into a Westergren tube.
b. Mix the blood gently to ensure uniform distribution of cells.

2. Centrifugation:

a. Place the Westergren tube containing the blood sample into the centrifuge rotor carefully.
b. Close the centrifuge lid securely to prevent accidents.
c. Set the centrifuge to a low speed (e.g., 1000 rpm) and allow it to spin for a specified time (e.g., 1 hour).
d. Higher speeds may be used for shorter durations to achieve similar results, depending on the experimental protocol.

3. Observation:

a. After centrifugation, carefully remove the test tube from the centrifuge.
b. Observe the test tube and mark the upper level of the sedimented red blood cells (erythrocytes).

c. Measure and record the height of the sedimented red blood cells.

4. Calculations:

Calculate the sedimentation rate using the formula:

$$\text{Sedimentation Rate} = \frac{\text{Height of Sedimented Red Blood Cells}}{\text{Time of Centrifugation}}$$

Typically, sedimentation rate is expressed in millimeters per hour (mm/hr).

Results:

- The sedimentation rate may vary depending on factors such as age, sex, and health condition of the individual, as well as the specific conditions of the experiment (e.g., centrifuge speed and time).
- Results are often interpreted in clinical settings to assess inflammation, anemia, or other blood disorders where sedimentation rate can be indicative of underlying conditions.

EXPERIMENT No. 12

PAPER CHROMATOGRAPHY OF AMINO ACIDS

Aim: To separate and identify amino acids in a mixture using paper chromatography.

Principle:

Chromatography is a technique used to separate individual components from mixtures. In paper chromatography, a homogenous adsorbent paper serves as the stationary phase, and a suitable solvent or solvent mixture serves as the mobile phase. The mixture's components are carried by the mobile phase as it passes through the stationary phase, with different affinities towards the stationary and mobile phases determining their travel rates. The retention factor (Rf) is calculated to compare the distances traveled by the solute and solvent.

$$\textbf{Retention Factor (Rf)} = \frac{\text{Distance the Amino Acid Migrated}}{\text{Distance the Solvent Migrated}}$$

The Rf value ranges between 0 and 1 and is dimensionless.

Materials:

Apparatus:

- Glass beakers
- Whatman filter paper (21 x 21 cm)
- Petri dishes
- Measuring cylinder
- Developing chamber

- Capillary tubes
- Pencils
- Stapler
- Drying oven
- Gloves
- Hood with wire for drying

Chemicals:

- n-Butanol
- Glacial acetic acid
- Distilled water (solvent mixture ratio 4:1:5)
- Amino acids: Tryptophan and Threonine
- Ninhydrin reagent
- 5 known amino acids (Leucine, Alanine, Phenylalanine, Aspartic Acid,
- Serine) (2 mg/ml in 10% isopropanol: 0.1M HCl)
- 1 unknown amino acid (2 mg/ml in 10% isopropanol: 0.1M HCl)

Procedure:

1. Preparation of Chromatography Paper:

a. Draw a line 3 cm from the bottom of the chromatography paper using a pencil.
b. Mark six circles, each 2 mm in diameter, and spaced 3 cm apart along the line. Label them as Leu, Ala, Phe, Asp, Ser, and Unkn.

2. Application of Samples:

a. Using a capillary tube, apply 2 µl of each amino acid sample onto the respective circles. Use a fresh pipette tip for each sample.
b. Allow the spots to dry, and repeat this process four times to ensure 10 µl of each sample is applied.

c. Allow the spots to dry completely.

3. Chromatography Setup:

a. Shape the paper into a cylinder and staple the edges to ensure they do not touch.

b. Place the cylinder in a chromatographic chamber under the hood Add the solvent mixture (n-Butanol: Glacial acetic acid: Distilled water in 4:1:5 ratio) to the chamber, ensuring the solvent line is 3 cm from the bottom of the paper.

c. Close the chamber and allow the chromatogram to develop for 60 to 90 minutes until the solvent front is less than an inch from the top of the paper.

4. Developing and Visualizing:

a. Remove the paper from the chamber and immediately mark the solvent front with a pencil.

b. Hang the paper under the hood to dry completely.

c. Spray the dried paper with ninhydrin reagent to visualize the amino acid spots.

d. Place the paper in a drying oven at approximately 100°C for 34 minutes to develop the Colour.

5. Measurement and Calculation:

a. Measure the distance traveled by the solvent and each amino acid spot.

b. Record the measurements and calculate the Rf value for each amino acid using the formula:

$$\textbf{Retention Factor (Rf)} = \frac{\text{Distance the Amino Acid Migrated}}{\text{Distance the Solvent Migrated}}$$

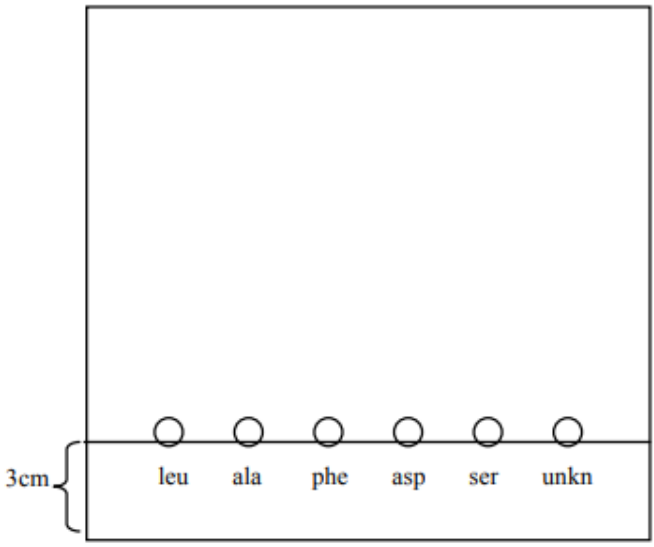


Fig A: Setup of Chromatogram

c. Make sure the line is 3cm from the bottom and the samples are at least 1 inch from the edge

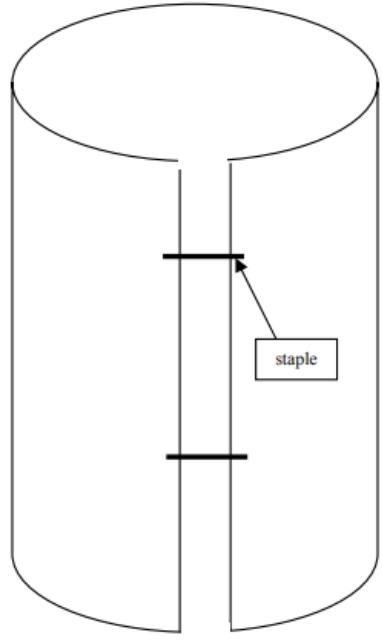


Fig: B: Stapling the Chromatogram

Observations:

Amino Acid	Distance Migrated (cm)	Rf Value
Leucine		
Alanine		
Phenylalanine		
Aspartic Acid		
Serine		
Unknown		

Results:

1. The distances moved by tryptophan and threonine are measured as …….. cm and .....cm respectively.
2. The distance moved by the solvent is ..... cm.
3. The Rf value of tryptophan is ……..
4. The Rf value of threonine is .....
5. The Rf values for the unknown mixture are …..... and .....

By comparing the Rf values of the unknown mixture with those of the known amino acids, it can be determined if the unknown sample contains tryptophan, threonine, or other amino acids.

Conclusion:

The Rf values of tryptophan, threonine, and the unknown samples were determined using ascending paper chromatography. The unknown samples were identified as tryptophan and threonine based on their Rf values.

EXPERIMENT No. 13

THIN LAYER CHROMATOGRAPHY (TLC) SEPARATION OF SUGARS

Aim: To separate and identify different sugars using thin layer chromatography (TLC).

Principle:

Chromatography is a technique used to separate and analyze bio-molecules from complex mixtures. In TLC, a type of solid liquid chromatography, a polar absorbent (stationary phase) is used along with one or more solvents (mobile phase). The mixture to be separated moves through the stationary phase due to capillary action. Compounds with higher affinity for the stationary phase move slower, while those with lesser affinity move faster. Thus, the components are separated based on their relative affinities towards the stationary phase.

Materials:

Chemicals

- Silica gel G (100 g)
- Mobile phase: Ethyl acetate, Isopropanol, Water, Pyridine (26:14:7:2)
- Sugars: Glucose, Rhamnose, Ribose, Xylose (100 mg each)
- Developing reagents: Aniline (1 g), Diphenylamine (1 g)
- Phosphoric acid (25 ml)
- Sodium acetate (2 g)
- Acetone (100 ml)

Apparatus

- Capillary tube
- Beaker
- Stirrer
- Chromatography chamber
- Glass plates
- Oven
- Spray gun

Glassware:

- TLC chamber (with lid) or a beaker that can fit the TLC plate

Other requirements:

- Micropipette
- Tips
- Hot air oven/Incubator

Procedure:

Preparation of TLC Plate and Loading of Standard Solution:

1. Draw two straight lines on the white surface of the TLC plate using a pencil: one 2 cm from the bottom and another 1 cm from the top.
2. Prepare a uniform paste of silica gel in distilled water and apply it to the plate to achieve an even thickness.
3. Spot 1 µl of each sugar sample and test sample along the bottom line using a micropipette, changing the tip for each sugar. Label the back of the plate and dry it.

4. Pour the prepared mobile phase mixture into the chromatography chamber and allow it to saturate for 30 minutes.
5. Place the TLC plate in the chamber such that it just touches the solvent surface. Cover the chamber.
6. Allow the solvent to rise until it reaches 3/4 of the plate, then remove the plate and mark the solvent front.
7. Air dry the plate for 15-20 minutes, then further dry it at 70°C for 2 minutes.
8. Spray the developing reagent onto the plate using a sprayer.
9. Measure the distance traveled by the developed spots from the origin line.

Calculation of Rf Values: Calculate the Rf value for each separated sugar using the formula:

$$\textbf{Rf value} = \frac{\text{Distance moved by compound}}{\text{Distance moved by solvent}}$$

Observations: Record the distances traveled by each sugar sample and the solvent front.

Results: Calculate and tabulate the Rf values for each sugar sample.

Precautions:

1. Read the entire procedure carefully before starting the experiment.
2. Allow all components to reach room temperature before beginning.
3. Change the micropipette tip for each sugar sample.
4. Wear gloves while handling reagents and TLC plates.

EXPERIMENT

No. 14

SEPARATION OF DNA MIXTURE BY AGAROSE GEL ELECTROPHORESIS

Aim: To separate and analyze a mixture of DNA fragments using Agarose gel electrophoresis.

Introduction:

Agarose gel electrophoresis is a widely used technique in biochemistry and molecular biology for the separation of DNA and RNA according to size. It is also used for quantification, purification, and analysis of nucleic acid fragments. The choice of gel matrix (Agarose or Acrylamide) and its concentration depends on the size of the nucleic acid molecules to be separated.

w/v % Gel type	Size of DNA fragments (Kb)
0.5 %	1 kb to 30 kb
0.7 %	800 bp to 12 kb
1.0 %	500 bp to 10 kb
1.2 %	400 bp to 7 kb
1.5 %	200 bp to 3 kb
2.0 %	50 bp to 2 kb

Principle:

Agarose gel electrophoresis separates DNA molecules based on size using an agarose matrix. DNA molecules, negatively charged

due to their phosphate backbone, migrate towards the positively charged anode under an electric field. The migration rate is inversely proportional to the size of the DNA fragments. Factors influencing DNA migration include the size of the DNA molecules, agarose gel concentration, applied voltage, DNA conformation, and the buffer used. Common buffers include TAE Tris **A**cetate_ EDTA (TAE) and Tris borate EDTA (TBE). Visualization of DNA is achieved using ethidium bromide staining and UV light.

Materials Required:

Chemicals:

- Agarose powder
- 1X TBE buffer
- Ethidium Bromide (5 mg/ml)
- Gel loading dye (Glycerol and orange dye)
- DNA samples
- 1 kb and 100 bp DNA ladder (DNA size standard)

Apparatus:

- Horizontal electrophoresis apparatus
- Power supply
- Glassware:
- Gel casting tray
- Comb
- Beaker

Other Equipment:

- Micropipette and tips
- UV transilluminator

- Orange filter for photography

Preparation of Stock Solutions:

1. TBE Buffer (10X, for 1 L):

- 108.0 g Tris base
- 55.0 g Boric acid
- 9.3 g Na2EDTA

Sterilize by autoclaving

2. TAE Buffer (10X, for 1 L):

- 302.5 g Tris base
- 71.4 g Acetic acid
- 18.6 g Na2EDTA

Sterilize by autoclaving

3. Ethidium Bromide (Stock Solution):

- Dissolve 10 mg ethidium bromide in 10 ml sterile distilled water and store at 4°C.
- Loading Dye (6X): 3 ml glycerol (30%) 25 mg bromophenol blue (0.25%) in 10 ml sterile distilled water.

4. Preparation of Agarose Solution for Gel Casting:

1. Measure out the required amount of agarose powder for a 1% gel.
2. Dissolve the agarose in TBE buffer by heating in a microwave until fully melted.
3. Cool the solution to lukewarm and add 4 µl of ethidium bromide.

4. Pour the agarose solution into a gel casting tray with a comb inserted to form wells. Allow the gel to solidify.

Protocol:

1. Gel Preparation:

a. Measure the required amount of agarose for a 1% gel.
b. Dissolve agarose in TBE buffer and heat until fully melted.
c. Add ethidium bromide and pour into a casting tray with a comb.
d. Allow the gel to solidify.

2. Gel Electrophoresis:

a. Place the gel in the electrophoresis chamber and cover with 1X TBE buffer.
b. Carefully remove the comb to create wells.
c. Mix DNA samples with loading dye.
d. Load DNA ladder into the first well and samples into adjacent wells.
e. Electrophorese at 95 Volts for 45 minutes.
f. Remove the gel and visualize DNA bands under UV light.

Observations: Record the distance traveled by each DNA fragment and compare it with the DNA ladder to determine fragment sizes.

Results: DNA bands appear as orange fluorescence under UV light. Photograph the gel for documentation.

Factors Influencing DNA Migration:

- Agarose Concentration: Higher concentrations for smaller fragments and vice versa.
- Molecular Weight: Migration rate inversely proportional to the logarithm of the molecular weight.

- DNA Conformation: Supercoiled DNA migrates fastest, followed by linear and relaxed circular forms.
- Applied Voltage: Higher voltage increases migration rate but may cause band distortion.
- Buffer Composition and Temperature: Optimal conditions ensure consistent migration rates.

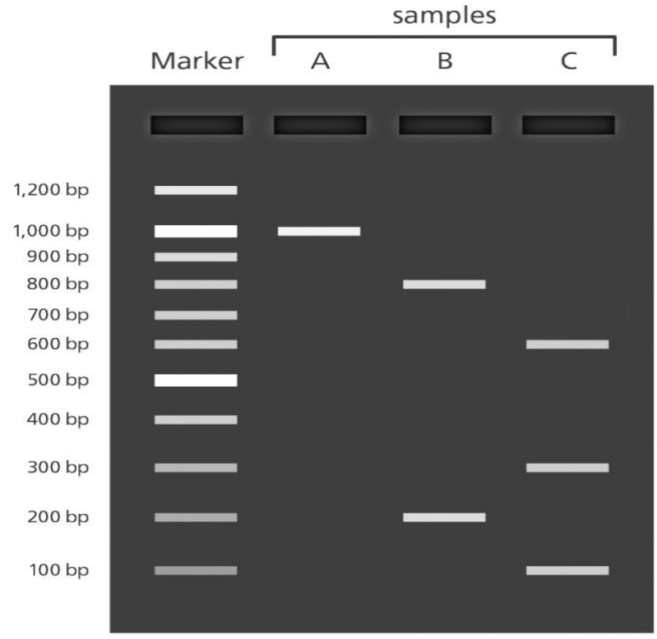


Figure 7: Result of Gel electrophoresis (This is sample result for reference)

Precautions:

- Read the entire procedure before starting the experiment.
- Allow components to reach room temperature before use.
- Wear gloves when handling reagents and agarose gel.
- Change micropipette tips between samples to avoid contamination.

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