MICROTOMY AND LYOPHILIZATION

Dr. Shama Afroze Baig
Head of Department (Microbiology)
Swami Shri Swaroopanand Saraswati Mahavidyalaya
Hudco, Bhilai, C.G.

Dr. Sanju Sinha
Assistant Professor
Department of Zoology
Government V.Y.T. PG Autonomous College
Durg.

I. MICROTOMY

Microtomy (micro-small, Tome means cut gr.) is a method for preparing very thin sections of tissue mounted on a glass slide to facilitate microscopic examination. Swiss anatomist Wilhelm His Sr. is the father of microtomy. To study the details of biological tissues, microorganisms and parasites, thin sectioning is required because most of these tissues are optically dense. For histological studies, tissues are preserved by embedding in paraffin wax.

II. MICROTOME

This is a Greek word meaning (Mikros-small, temnein means to cut). Tissue embedded in a paraffin block of predetermined thickness with a machine-mounted microtome knife, glass or diamond blade. A microtome is a mechanical device that helps to achieve a uniform thin slice of biological tissues for various microscopic studies. The microtome has a knife, a stand and a specimen holder.

Principle

The instrument used to cut is a microtome. It has spring-balanced teeth, a front mechanism wheel connected to a micrometer screw, which in turn turns and moves the target, a block of paraffin, a predetermined distance until it contacts the knife, knife or blade. The sample moves vertically past this cutting surface and a fabric sectionis created.

III. INSTRUMENTATION

A microtome consists of base, knife and sample holder.

1. **Base (Microtome Body)**

2. **Knife Mount and Blade:** Different types of knives are used with different microtomes. A wedge knife (type C) is used for routine use. It is flat on both sides. The size varies from 100 mm to 350 mm. Microtome knives are made of high-quality high-carbon steel or steel with a hardened tip. Knife hardness is essential for good tissue cuts.

3. **Tissue Holder:** Some microtome's have more functions like a table clamp to hold the machine in place. Some devices are manually operated, while others are automatically operated, so the special features of this microtome are different.

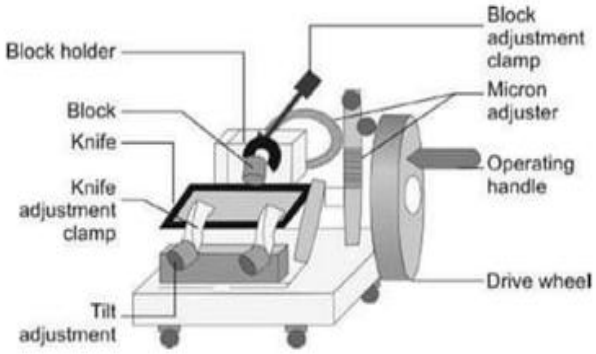

Figure 1:

Types of Microtomes

There are several different types of microtomes, depending on the tissue and purpose, and many have a multi-functional role. There are several different microtomes available:

- **Rotary Microtome** - to cut paraffin-embedded sections
- **Sledge Microtome** - to cut cheloidin-embedded sections
- **Cryomicrotome** - to cut non-embedded frozen sections
- **Cryostat** - to cut frozen sections
- **Ultrathin Microtome** - to cut sections for electron microscopy
- **Vibrating/oscillating microtome** - for difficult biological samples

- **Saw microtome** - for hard materials such as bone, teeth
- **Laser microtome** - for non-contact cutting

1. **Rotary Microtome:** This is the most commonly used microtome. It is used to cut blocks embedded in paraffin. It can also be used on cryostat frozen parts and also on resin coated cases. To cut, the head of the microtome containing the block is turned over the blade.

2. **Sliding Microtome:** In this microtome, the sample is placed in a fixed holder (shuttle), which then moves back and forth along the blade. During the operation, the pressure on the sample can be reduced. A typical section thickness that can be achieved with a sled microtome is 1-60 m

3. **Cryomicrotome:** This is used to cut frozen specimens. The reduced temperature allows to increase the hardness of the sample, which allows the preparation of semi-thin samples. However, to optimize the resulting sample thickness, the sample temperature and the knife temperature must be adjusted.

4. **Ultra-Thin Microtome:** This is a microtome primarily used in electron microscopy. This makes it possible to produce very thin parts. This microtome uses a diamond knife (preferred) and a glass knife. To collect sections, they are floated on liquid during sectioning and carefully picked up on suitable grids for TEM viewing.

5. **Vibrating/Swinging Microtome:** This is a small microtome with two swinging arms. Parts are cut. The other eats through the tissue block. It is limited to cutting small soft blocks because it uses a spring to cut.

6. **Sliding Microtome:** This is a microtome with an unusual design where the blade moves across the block instead of the block. It is good for cutting celloidin, although it can also produce good paraffin sections.

Procedure

The main steps in the preparation of histological slides by microtomy are:

- **Fixation:** To prevent the disintegration of the cell and keep it alive.
- **Processing:** Drying, cleaning and soaking.
- **Cutting/sectioning:** Cut very thin sections from the wax embedded block.

- **Staining:** Create visible contrast.
- **Mounting:** Creating a permanent slide.

1. **Fixation:** Fixation is the protection of biological tissues against degradation caused by autolysis or decomposition. Biological tissue samples are fixed to preserve the cells/tissues in as natural a state as possible. Chemical fixing agents are very carefully selected substances whose properties must meet many criteria. Even the most careful mounting will alter the sample to some extent and may cause artifacts. Artifacts are tissue structures or features that interfere with normal histological examination, ie. pigments formed by fixatives. The choice of fixation method and special fixation means may depend on the subsequent processing steps. Fixation is a reaction between the fixing agent and the proteins in the sample that form a gel, holding everything together in life.

   The purposes of fixation are:

   - To prevent autolysis and bacterial attack.
   - To fix the fabrics so that they do not change their volume or shape during processing.
   - Prepare the tissues and leave them in a condition that allows the sections to stain clearly.
   - Leaves the tissues in a vital state.

IV. CHEMICAL FIXATION

Biological structures are preserved as close as possible to the state of living tissue. This requires a chemical fixative that can stabilize proteins, nucleic acids and tissue mucous substances, making them insoluble. Some chemical fixatives include: (1) acetic acid, (2) formaldehyde 10%, (3) ethanol, (4) glutaraldehyde, (5) methanol, (6) picric acid, and (7) osmic acid (osmium tetroxide). Tissue samples should be cut into small cubes approximately 1cmx1cmx1 cm and placed in fixative (i.e 10% formaldehyde/ formalin) for 24-28 hours.

V. FROZEN FIXATION

Small pieces of tissue (usually 5 mm x 5 mm x 3 mm) are placed in a cryoprotective medium. 2. They are then flash frozen in isopentane (alkane) cooled with liquid nitrogen (flash cooling for preservation). 3. The tissue is then cut on a cooling microtome (cryomicrotome, which will be discussed

later). 4. The parts are then fixed by immersion in a specific fixing agent or set of fixings for a precisely controlled time.

Processing

This is the embedding of tissues in a solid medium, strong enough to support them and give them enough rigidity to cut thin sections, yet soft enough not to damage the knife or tissues. The processing steps are:

- **Drying** (removal of water)
- **Cleaning** (removal of alcohol)
- **Immersion** (preparation of paraffin wax block)

VI. TISSUE PROCESSING STEPS

Drying/ Dehydration: Removal of water, dehydration removes fixed substance and water tissues and replace them with a dryer. Samples are dehydrated in an increasing ethanol series: 10%, 20%, 50%, 70%, 95%, and 100% absolute (about 30 min each). Common dehydrating agents are: ethanol, methanol and acetone. Tissues can be preserved without damage and stored indefinitely in 70% ethanol.

Clearing: This process uses an organic solvent such as xylene to remove the alcohol and penetrate the paraffin wax.

Common cleaning agents include:

(1) xylene, (2) toluene, (3) chloroform, (4) benzene, and (5) propylene oxide.

Embedding: Here the tissue is first placed in warm paraffin wax at 58oC for one hour. Wax penetrates it and replaces the xylene, and the tissues are surrounded by a medium like paraffin wax (to form a block). As the wax hardens, it provides support and strength to the tissue during cutting. The general purposes of immersion are:

- To improve ribboning
- To increase hardness
- To lower meltingpoint
- To improve adhesion between samples

Wax Mounting is done in molds/cases such as:

- Paper boat mold,
- Metal boat mold,
- Removable mold, and
- Base mold.

Sectioning is the preparation of thin slices of wax-covered tissues with a microtome. The sections are 5m thick in light microscopy and 80-100 nm in electron microscopy. A microtome is a mechanical instrument used to cut biological specimens into very thin sections for microscopic examination. Thin sections of paraffin-embedded tissue are cut with a microtome. Most microtomes use steel (diamond blade in ultramicrotomes). They are used to prepare animal or plant tissue sections for histology. After removing the parts, they are placed in hot water (to smooth them). They are then taken from below onto a glass chute. The slide with the cut is left to dry at 37^{o}C, so that the part can be fixed.

Tissue Sections Transverse Section (T.S) A horizontal section taken in a plane at right angles to the longitudinal axis of the subject's body. T.S goes between the side ends. It is usually relatively shorter. The number of possible cross-sections of the sample is relatively larger. A vertical cut that is cut along the longest axis of the object. Longitudinal Section (L.S) L.S passes through the front rear axle. It is usually relatively longer. The number of possible longitudinal sections passing through the sample is relatively smaller.

Staining: Staining refers to the use of stains to visualize cells and/or cellular structures and improve the contrast of the microscopic image. Mounted sections are processed with appropriate histological staining. There is very little variation in the color/tone of biological tissues when viewed under a microscope. Staining of biological tissues is done both to increase tissue contrast and to emphasize some interesting features. Staining of paraffin embedded sections is done as follows:

Deparaffinization: Wax is removed from the section by applying xylene. Rehydration: The sample is rehydrated with a decreasing series of ethanol: 90%, 70%, 50%, 20%, 10% and distilled water (about 30 minutes each).

Hematoxylin and Eosin Staining

- The tissue is first counterstained with hematoxylin for 10 minutes (this stain gives the nuclei a blue color).
- The tissue is then washed with distilled water and then immersed in acid alcohol until only the nuclei are stained blue.
- The slide is washed again with distilled water and neutralized with alkaline tap water.
- It is washed again with distilled water and then stained with eosin for 10 seconds only to stain the cytoplasm, extracellular matrix, collagen and erythrocytes.

Dehydration: The sample is re-dehydrated in an increasing ethanol series: 10%, 20%, 50%, 70%, 95% and 100% ethanol (about 30 minutes each).

Clearing: The sample is clarified for 15 minutes in a clarifying agent such as xylene to remove any traces of alcohol and increase the refractive index of the sample to make it more transparent.

Mounting: Mounting is done to preserve and support the stained section for microscopic examination. To mount a slide:

- Apply one drop of DPX Mountant solution to the tissue section.
- Keep the lid at a temperature of 45 o C so that the drop spreads along the edge of the glass.
- Leave and let the medium spread slowly

Applications

For freehand parts, the thickness of the part cannot be adjusted, it can be thick or thin, as well as oblique. In addition, the entire material cannot be received in parts, because defective parts must be removed. Various applications of the microtome include:

- Traditional histological technique
- Cryosection technique
- Electron microscopic technique
- Botanical microtome technique: (Sledge microtome is required for hard materials like wood. These microtomes have heavier blades and cannot cut as thin as a regular microtome.)

When using a microtome to cut, the sections are of uniform thickness. They can be made in any desired thickness (10 or 15 or 20 n etc.). Parts are not tilted. The whole tissue can be preserved in sections - this is especially necessary for the study of the development of plant organs. However, microtome cutting is a long and laborious process.

Lyophilization

Lyophilization is an exciting process used in many fields, especially in pharmaceuticals, biotechnology, and food preservation. Freeze drying is a complex dehydration method that involves removing water or solvent from a material by changing it from a frozen state to a gas without a liquid phase. This is achieved by sublimation, which occurs when ice or a solvent changes from a solid to a vapor without becoming a liquid.

Definition

A process in which a sample is first frozen and, after freezing, most of the water and solvent system is removed by sublimation and desorption to limit biological and chemical reactions at a specific storage temperature.

Also known as freezing. , it is often used in the food industry, for the preservation of biological samples and in the process of pharmaceutical applications, which is a suitable drying technique for heat-sensitive samples. It is a process in which water, which is under low pressure in the form of ice, is removed from the sample by sublimation. This process has found many applications in the production of high quality food and medicine.

Principle

The freeze-drying process consists of freezing the product to turn the water in the product into ice form, the ice is directly sublimated into water vapor in a vacuum, and then the water vapor is removed.

History

Freeze-drying dates back to ancient times, when civilizations used primitive freeze-drying processes to preserve food and medicinal plants. German histologist Richard Altmann made a decisive breakthrough in 1906 when he succeeded in freeze-drying bacteria for preservation and then reviving them. This experiment created a framework for further development of the process.

During World War II, lyophilization was used to preserve blood plasma for the military, which led to further developments. The pharmaceutical industry realized the potential of lyophilization for the preservation of heat-sensitive drugs in the following decades, and commercial lyophilization became a reality in the 1950s.

Equipment and Process

The device is known as a freeze dryer.

Freeze Dryer Components and Functions

1. **Condenser:** A condenser is an important component of a freezer that converts the water vapor produced during the freeze drying process back into water steam to freeze the vacuum system lowers the pressure in the freeze dryer and forces the frozen water in the material to flow into steam. That water vapor is then directed to a condenser where it rapidly cools, condenses and returns to a solid state. The condenser is critical for removing water from the system and preventing it from accumulating.

2. **Suction System/ Vaccuming:** A vacuum system is important in the freeze-drying process, which lowers the ambient air pressure in the freeze-drying chamber. The boiling point of water is lowered by lowering the pressure so that the frozen water in the material can change directly from solid to vapor without passing through the liquid phase. This sublimation process successfully removes water from the material, leaving a dry and stable result.

3. **Shelf Heater:** Shelf heaters are responsible for providing controlled heat to the material during the freeze drying process. The frozen material is treated with gentle heat during the first drying cycle, which facilitates the sublimation and transformation of the ice into steam. Shelf heaters maintain the necessary temperature levels during several drying cycles to achieve an efficient and effective freeze-drying process or lyophilization process.

Lyophilization Process

1. **Loading and Pre-Freezing:** Pre-freezing of the dried substance is the first step in the lyophilization process. Pre-freezing is the process of lowering the temperature of a material significantly below its freezing point to ensure the formation of small, homogeneous ice crystals. This step is critical because it prevents the formation of large ice crystals that can damage the

structure of the material. After the material is pre-frozen, it is loaded onto the shelves in the freeze-drying room. To achieve efficient heat and mass transfer during subsequent drying cycles, it is important to ensure uniform loading.

2. **Primary Drying Cycle:** The first stage of the lyophilization process is the primary drying cycle. The freeze dryer gently heats the frozen material at this stage. This heat causes the frozen water to sublimate, turning it from a solid to a vapor. The vapor is then removed from the material and directed to a condenser where it is cooled and solidified. Pre-drying removes a significant amount of water from the material.

3. **Secondary Dry Cycle:** The material still contains bound water after the initial dry cycle, which cannot be easily removed by sublimation. This problem is solved by a secondary drying cycle, which uses a slightly higher temperature andlower pressure. This controlled environment allows reman-ing bound water to desorb, turning it into vapor and removing it from the substance.

4. **Final Drying Cycle:** The last drying cycle completes the freeze-drying process. This implies raising the temperature slightly higher than in previous cycles to ensure complete removal of excess water or solvent. The duration of the final drying period depends on the substance to be lyophilized and its individual needs.

5. **Unloading of the Products:** The dried and stable material is available for disassembly after the lyophilization is finished. This step must be handled carefully to avoid damaging the freeze-dried product. The material is removed from the freezer shelves and packaged for storage or further processing.

Applications of Lyophilization

Lyophilization, commonly known as lyophilization, is a versatile method with many applications in many fields, e.g. in pharmaceutical, food, biotechnology and scientific industries.

Pharmaceutical Industry

Lyophilization has revolutionized the pharmaceutical industry by enabling the preservation and stabilization of sensitive drugs and biological substances. Its

uses in industry are numerous and crucial to pharmaceutical efficacy and shelf life.

Retention of Vaccines

One of the most important applications of lyophilization is the storage of vaccines. Vaccines can be stored and shipped more easily by freeze-drying, especially in areas where refrigeration is limited. Lyophilization ensures vaccine stability and efficacy, contributing to global immunization efforts.

Biological Agents and Antibiotics

Some antibiotics and biological agents, such as proteins and peptides, are sensitive to temperature and humidity. Lyophilization is critical to preserving these chemicals, preserving their potency and utility, and enabling widespread use in medical therapy.

Use of Lyophilization in Gene Therapy

Gene therapy is a modern form of treatment for diseases caused by genetic abnormalities and faulty genes. Gene therapy is the insertion of genetic material into a patient's cells to repair or replace faulty genes. Lyophilization is critical in this process because it preserves and stabilizes the gene therapy vectors. These vectors act as carriers to deliver therapeutic genes into the patient's cells. The biological activity of the vectors is preserved by lyophilization, which enables optimal gene transport and therapeutic effects.

In the Food Industry

Freeze drying is also useful for the food industry, which uses it to improve flavours, extend shelf life and create new delicious experiences.

- Making instant coffee and tea
- Foods and freeze-dried fruits
- Biotechnology and research

Preservation of Microorganisms

Freeze-drying/Lyophilization can be used for long-term preservation of microorganisms such as bacteria and fungi. This preservation method allows researchers to preserve a variety of cultures for research, quality control, and production.

Enzymes and Proteins are Lyophilized

Enzymes and proteins are important macromolecules used in many fields, including medicine, biotechnology, and food processing. Freeze drying preserves their activity and stability, which allows them to be stored, transported and used in many different applications.

Advantages of Freeze Drying

- Long shelf life and better stability
- Retention of biological activity
- Improved solubility.
- Shipping and storage costs are reduced.

Technical and Economic Challenges

- Complex equipment and processes
- Sensitivity of biological material
- Expensive and time consuming

VII. CONCLUSION

In conclusion, lyophilization, also known as freezing, is a complex process used in many ways in the pharmaceutical, biotechnology and food industries. Its ability to preserve sensitive materials, improve stability and extend shelf life has revolutionized several industries. However, this complex approach is affected by several factors, including formulation problems, freezing methods and climatic conditions. Adopting sustainable practices and embracing breakthroughs in freeze-drying technology will certainly determine its future impact, leading to more personalized treatments, advances in nanotechnology and better food preservation techniques. As lyophilization evolves, it continues to be an important tool for advancing research, treatment, and product development in a variety of industries.

References

[1] Lena T. Spencer, John D. Bancroft, in Theory and Practice of Histological Techniques (Sixth Edition), 2008
[2] Lena T. Spencer, John D. Bancroft, in Bancroft's Theory and Practice of Histological Techniques (Seventh Edition), 2013
[3] Tortora, G. J., Funke, B. R., & Case, C. L. (2021). Microbiology: An introduction. Pearson Education Limited.

[4] Willey, J. M., Sandman, K. M., Wood, D. H., & Prescott, L. M. (2019). Prescott's microbiology (11th ed.). McGraw Hill.
[5] Madigan, M. T., Bender, K. S., Buckley, D. H., Sattley, W. M., Stahl, D. A., & Brock, T. D. (2022). Brock Biology of Microorganisms. Pearson.
[6] https://en.wikipedia.org/wiki/Freeze_drying