Specimen Preparation for Electron Microscopy

- ✓ **Basically similar to that of light microscopy, with some variations.**

- **Chemically killed and fixed**
- **Infiltration**
- **Embedding**
- **Cut into ultra thin sections on ultra microtomes**
- **Staining**

8 Steps

1. Fixation
2. Block Staining
3. Dehydration
4. Infiltration
5. Polymerisation
6. Sectioning
7. Mounting
8. Section Staining

1. Fixation

Also called Osmication – use of Osmium tetroxide- widely used.

(other fixatives include aldehydes, acrolein, potassium permanganate, etc.)

Penetration rate is slow, but reacts rapidly with proteins, unsaturated fatty acids and phospholipids. So the tissues must be cut into very small pieces.

1. Fixation - Fixatives

Osmium tetroxide - provides best electron constrast, although it has no effect on carbohydrates such as glycogen. It does not harden tissues, therefore ultra thin sectioning is possible; neither shrink nor swell fixed tissues.

Alehydes – preserve structure excellently, but allow lipids to be completely extracted by the dehydrating alcohol. They also preserve glycogen, but not good in providing electron contrast.

1. Fixation - Fixatives

Osmium tetroxide – Disadvantages

1. Allows glycogen to escape during subsequent procedure
2. Does not react significantly with nucleic acids
3. Penetrates tissues very slowly due to low diffusion rate

These can be overcome by using Aldehyde as primary fixative and Osmium tetroxide as secondary fixative.

1. Fixation - Fixatives

Glutaraldehyde– Primary fixative

1. Non-coagulant.
2. Solution of 4% glutaraldehyde in phosphate buffer (pH 7.0)
3. Specimen is prefixed in this for 2 hrs. to overnight

Osmium tetroxide - secondary fixative

1. Specimen postfixed in 2% OsO_4 prepared in phosphate buffer (pH 6.8) for 1-2 hrs.
2. It provided positive electron contrast to membranes and preserves unsaturated lipids

1. Fixation - Fixatives

Use of Buffers

- To maintain the pH of the fixative solution at the physiological value,
- To prevent the shrinkage and swelling of the tissue by osmotic pressure effects,
- They are non-toxic, non-reactive chemically with the fixative, do not precipitate out with change in pH.
- Eg. Michaelis Barbiturate buffer, Phosphate, Citrate and Acetate buffers.

1. Fixation – Fixation Technique

1. Two stage fixation procedures

- Glutaraldehyde buffered with phosphate followed by Osmium tetroxide – almost universally used.
- Glutaraldehyde – concentration 1-6% in

  0.05 to 0.1M buffer, pH 6.8 – 7.6;

  time 1-3 hrs.

  After primary fixation, Glutaraldehyde is washed out thoroughly.

  Secondary fixation is done at room temp.

1. Fixation – Fixation Technique

2. Single stage fixation procedure

- Palade's fixative is used (1% osmium tetroxide made up in 0.1M sodium barbiturate-sodium acetate buffer).
- Fixative is used in ice-cold conditions or at about $4^{O}C$.

2. Block Staining

For rendering the fine structure visible in electron microscope by certain components of it to attract heavy metal ions and thus to scatter electrons differentially.

Uranium, iron, bismuth and potassium permanganate solution are used following osmium fixation and before dehydration.

1% phosphotungstic acid (PTA) dissolved in final dehydrating alcohol gives very intese staining of collagen.

3. Dehydration

Aim - replace all free water in the specimen with a fluid which is miscible with both water and the embedding monomer.

Dehydrating agents: Ethyl alcohol, Methyl alcohol, Acetone, Methyl cellosolve

Ethyl Alcohol – most commonly used (no hardening of tissues, makes tissues brittle for subsequent ultra-thin sectioning)

Acetone – hardens the tissues

Process – A series of mixtures of water and dehydrating fluid of decreasing water concentration.

3. Dehydration

Refinement of Dehydration – pass the tissue from the last grade of alcohol to an intermediate medium, a fluid completely miscible with both alcohol and resin monomer.

Generally used is 1,2 epoxypropane (EPP) also called proplylene oxide.

4. Infiltration

Depends on choice of resin for the final embedding.

Early type – butyl methacrylate.

- ✓ **Good cutting properties when polymerised**
- ○ **Produces gross artefacts in the tissue due to shrinkage**
- ○ **Unstable in the electron beam and evaporates when irradiated. (advantageous for producing contrast)**

Now a days -Cross linked Epoxy Resins

- ✓ **Hardly no shrinkage during polymerisation**
- ✓ **Stable in the electron beam, sections can be examined without a supporting film.**
- ○ **Difficult to cut into ultra thin sections**
- ○ **Need to be stained to provide increased contrast**

5. Embedding - Media

Harder embedding medium, harder than paraffin wax is used.

Epoxy resins like Epon, Araldite, Maraglas

These chemicals are carcinogenic, cause skin reactions

Materials are embedded in these epoxy resin and polymerised to from the hard block.

Epoxy Resin

- **A family of thermosetting synthetic resin**
- **Characteristic light yellow or honey colour**
- **Mixes well with suitable curing agent**
- **On heating polymerise irreversibly into cross linked yellow brown solids.**

Other embedding medium is 'Vestopal-W' good for plant tissues.

5. Embedding - Procedure

- Tissue blocks remain in the vial.
- Embedding fluids are changed as in the dehydrating procedure.
- Final dehydrating alcohol + resin ➡ full strength resin mixture.
- Soak for some hours, change resin. Repeat several times.
- Resin becomes viscous after few hrs at room temperature.
- Final infiltration should be over night – medium is almost too viscous to pour.

5. Embedding - Procedure

- Mould – determined by the chunk on the microtome.
- 7-5mm in diameter and about 15mm long.
- Cast in a mould made of gelatin. (blocks are prepared in gelatin drug capsules)
- Tissue piece is placed centrally at the rounded base of capsule.
- Capsule filled with a low viscosity catalysed resin mixture, closed and placed in over at 60OC, left for 48 hrs to polymerise completely.
- Gelatin capsules are placed in hot water to remove capsule.
- Now the block is ready for trimming.

5. Embedding - Procedure

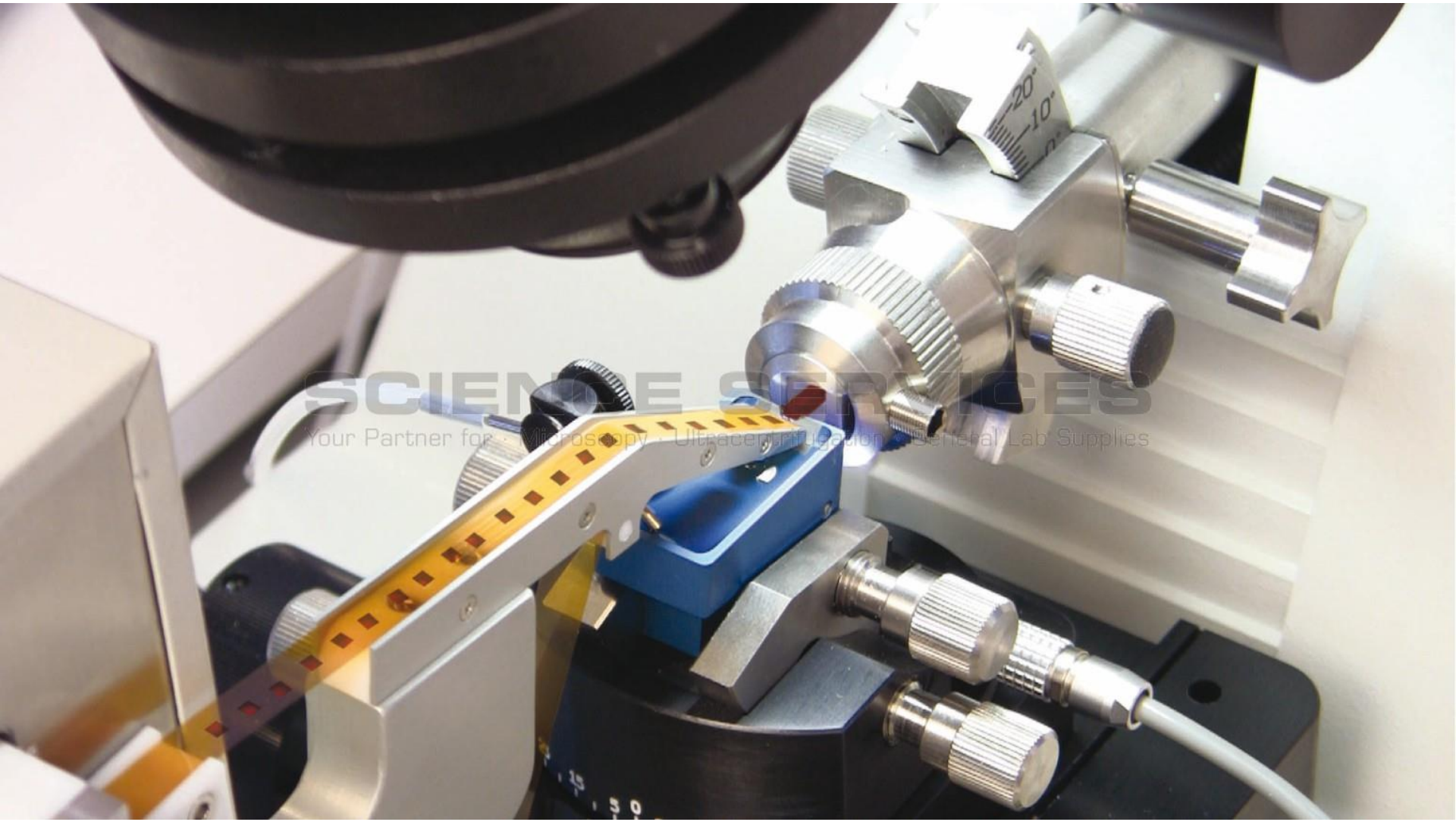
- Block mounted on the chunk under a stereo binocular X5 microscope.
- The rounded end of the block is trimmed free hand to four sided truncated pyramid of 45^{O} angle and 0.5mm square space at tope.
- Further trimming is done by cutting horizontally across the top of the block with a new razor blade.
- Tissue is exposed horizontally, further four cuts made at 45^{O} to the block axis to give a square top face (1mm) size; ready for cutting thick (0.5mm) sections.
- Block making machines are available now.
- Material is ready for sectioning with ultramicrotome and staining.

6. Ultramicrotomy

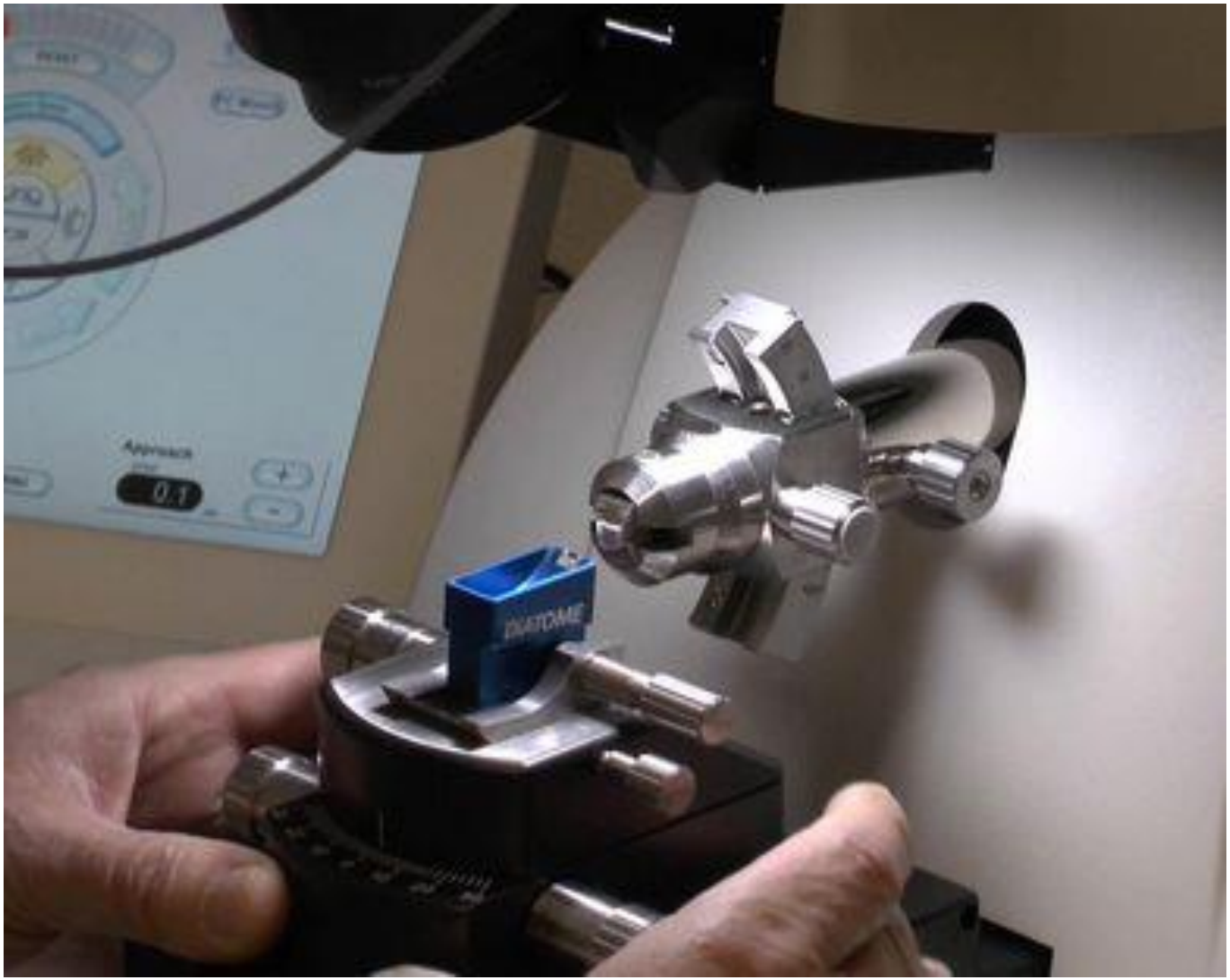
- **Sections of ultra thin dimension are required – electrons have low penetrating power.**
- **Sections of 60-100nm are required for examination under TEM.**
- **This is achieved by glass or diamond knife (for high quality sections) fitted to ultramicrotome.**

Ultramicrotome

Ultramicrotome

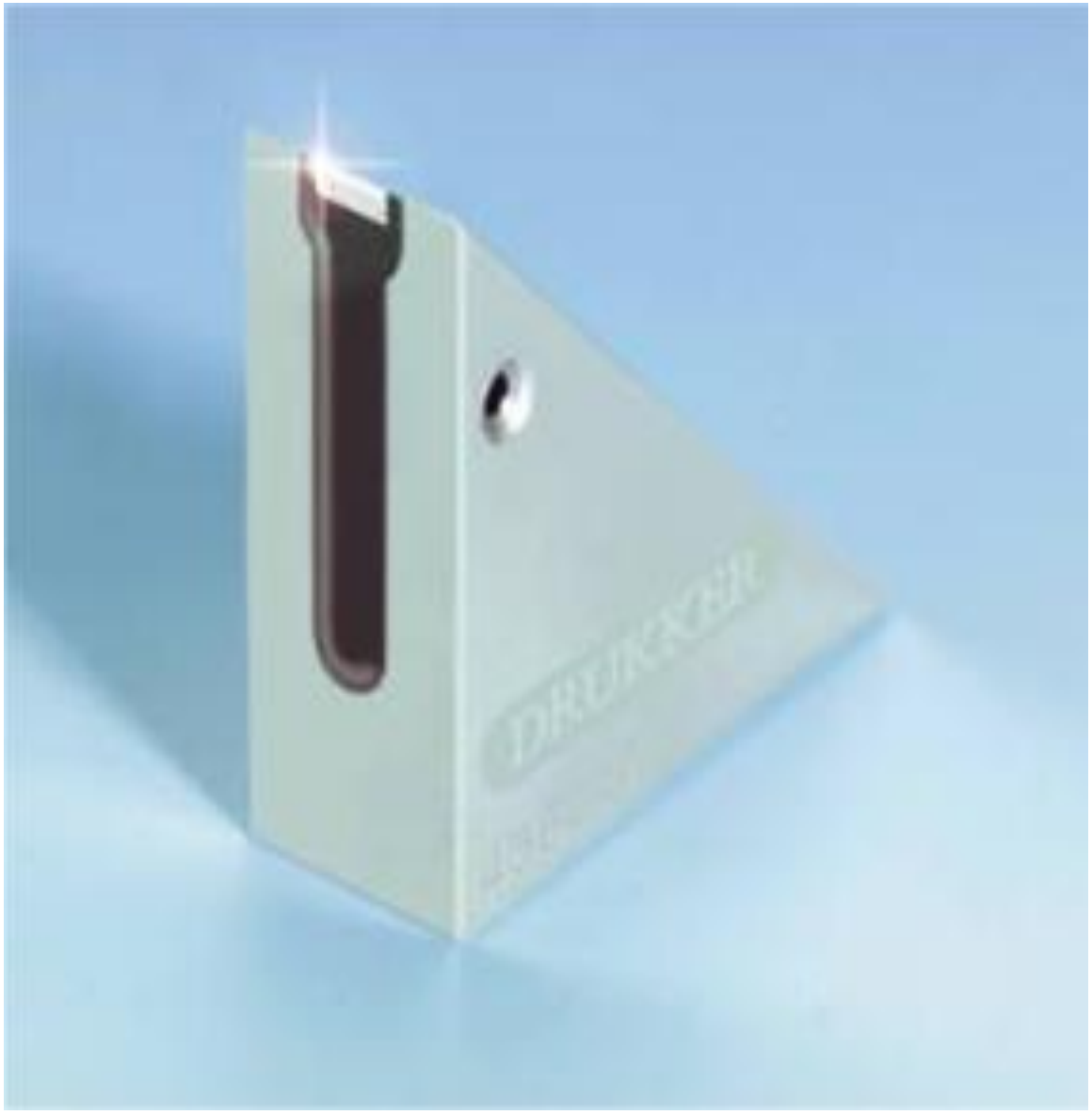


Ultramicrotome



Ultramicrotome

Ultramicrotome



Cryo microtome