TEM specimen preparation

fixation

embedding

sectioning

staining

FIXATION

- ***Goal***: Stabilize "normal" ultrastructure via chemical or physical preservation of specimen

  - minimum alteration from living state
  - protect against disruption during embedding and sectioning.
  - minimize shrinkage or swelling
  - rapid penetration

"Standard" EMBEDDING PROTOCOL (after glutaraldehyde fixation)

1. Millonig's Phosphate buffer (0.15M, pH 7.2-7.4).	10 min
2. Phosphate buffer (**Rinsing**)	10 min
3. Phosphate buffer	10 min
4. 1% OsO_4 in phosphate buffer (**Fixation**)	1 hr
5. Buffer	10 min
6. Buffer	10 min
7. 30% acetone (**Dehydration**)	10 min
8. 60% acetone	10 min
9. 90% acetone	10 min
10. 100% acetone	10 min
11. 100% acetone	10 min
12. 100% acetone	10 min
13. 100% acetone	10 min
14. 3:1 acetone:Resin * (**Infiltration**)	1 hour+
15. 1:1 acetone:Resin	overnight
16. 1:3 acetone:Resin	1 hour +
17. Undiluted resin	1 hour +
18. Embed in undiluted resin -**polymerize** at 60°C	18-24 hours

Tissue may be placed under vacuum for steps 15-18 to enhance resin penetration.

*Poly/Bed 812, Polysciences, Warrington, PA

FIXATION

- Coagulant fixatives –(i.e.ethanol or acetone) unsuitable for EM.
- Noncoagulant fixatives required.
- Additive fixatives (fixatives that become part of the protein structure) provide best ultrastructure.
- Glutaraldehyde - standard EM primary fixative.

Factors that affect fixation quality

- **pH**: Cell cytoplasm has a pH of ~7.0, nuclei have a pH of ~7.6-7.8. Different parts of the cell vary in pH.
- Average pH for animal tissues is ~7.4 (use pH of 7.2-7.4)
- Protozoa, invertebrates, and embryonic tissue may need slightly more alkaline - up to pH 8.0.
- Plant tissues buffered in range of 6.8-7.1

Factors that affect fixation quality

- **Buffer type**: Buffer type can affect the membrane appearance, cytoplasm granularity, increase or decrease the amount of protein extraction, & affect the staining & sectioning.
- Maintain pH
- Suitable osmolarity (minimize swelling or shrinkage)
- Suitable ionic concentration (minimize precipitation or extraction of components).

Phosphate buffers

- Most commonly used buffers
- Cheap, "physiological", non-toxic.
- Good buffer, but they are more prone to precipitates during processing.
- Cannot add calcium to fixative.
- May become contaminated with mold upon prolonged storage.
- Sorenson's or Millonig's PO_4 buffer formulations both work fine.

Cacodylate buffers

- Sodium salt of cacodylic acid used. pH adjusted with HCl.
- Easy to prepare, stable for long periods (does not support growth of microorganisms).
- Calcium may be added (enhanced membrane preservation)
- Contains ARSENIC. Do not inhale dust when weighing and avoid contact with skin.

Organic buffers

- **HEPES, PIPES**: Often used in tissue culture. Work well for EM.
- More expensive, tissues may stain less intensely.
- Can add calcium to fixatives.

Factors that affect fixation quality

- **Tonicity** (osmolarity)
- Osmol = Number of particles in 1 gr molecular weight of undissociated solute.
- NaCl = 2, glucose = 1, Na_2SO_4 = 3.
- Osmolarity = Osmols/liter; An isotonic 0.9% NaCl solution (.154 M) has an osmolarity of $\approx$ 310 mOsm - .154 M x 2 Osm = .308 Osm

Osmolarity

- Affects cell shrinkage or swelling, extraction of components, penetration rate of fixative.
- fixative is isotonic with a cell if the cell neither swells or shrinks.
- Empirically observed that fixatives that are calculated to be isotonic do not prevent cell swelling.
- In practice, most EM fixatives are slightly hypertonic

Factors that affect fixation quality

- **Fixative concentration**:
  - low concentrations generally require longer fixation times.
  - Longer fixation usually results in extraction of some components.
  - High concentrations destroy enzyme activity or alter antigens and also damage fine structure.

Factors that affect fixation quality

- **Temperature & time of fixation**.
  - higher temperatures generally increase rate of penetration and fixation.

  - Some cell components or tissues may be best preserved at 4C, RT, or physiological temperature (~37C)

  - Long durations (more than 24 hrs) usually detrimental to fine structure (extraction of components, artifacts).

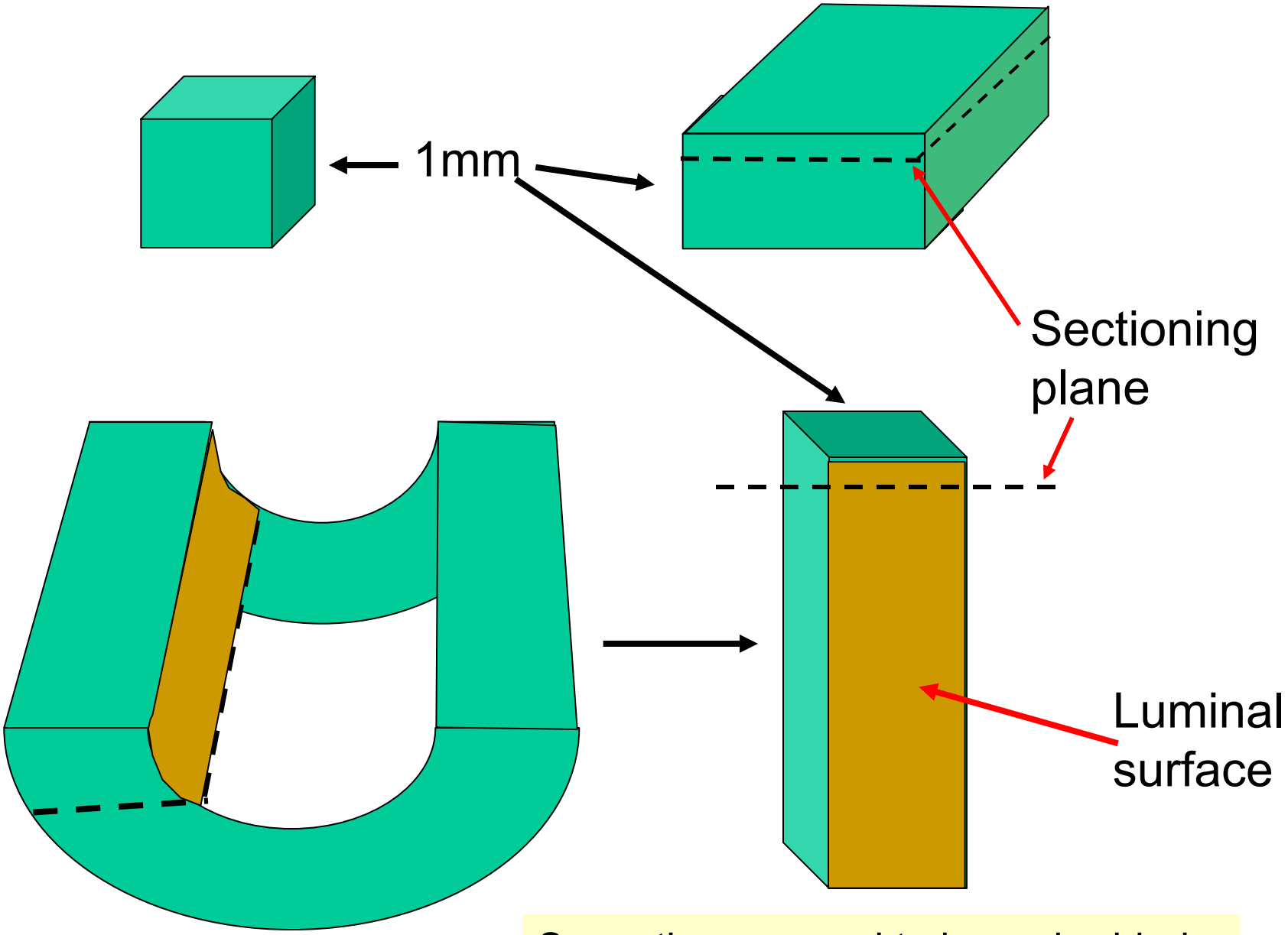
Factors that affect fixation quality

- **Specimen size**:

  - Important for fixative to penetrate rapidly and stabilize structures.

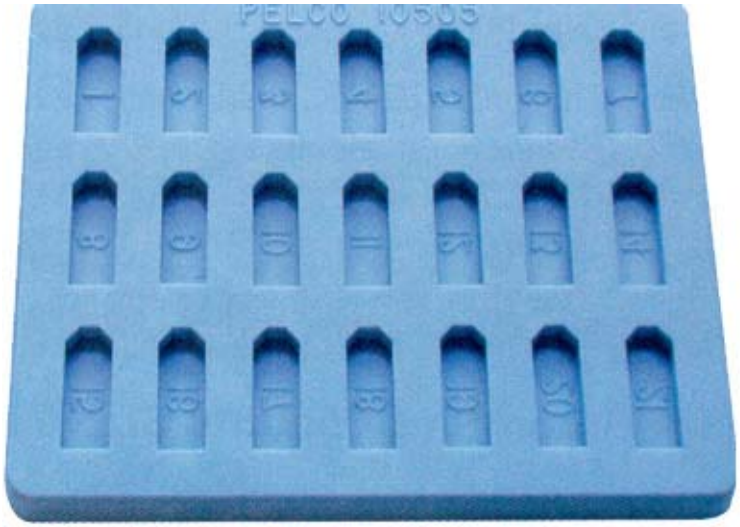
  - $1mm^3$ is considered to be the maximum workable size for good fixation of samples immersed in fixative. Smaller than $1mm^3$ for the very best fixation.

  - Large tissue blocks lead to poorly fixed regions in the center of the tissue.

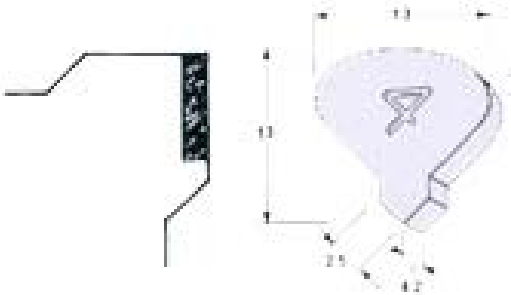


Some tissues need to be embedded in a manner that maintains orientation

Polyethylene embedding capsules



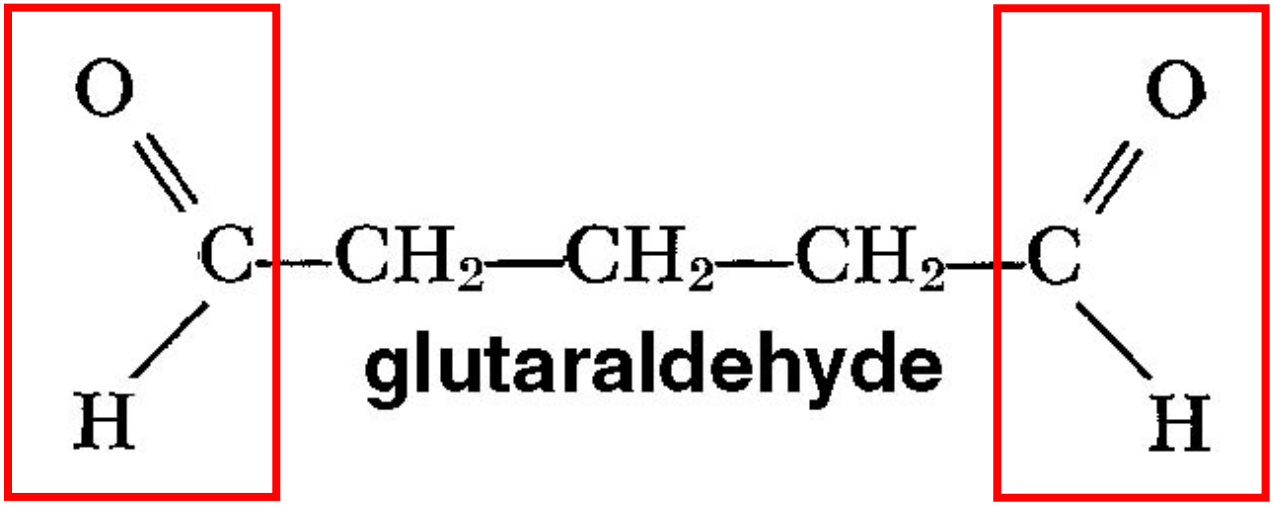
Chien Embedding Mold



Flat embedding molds for maintaining tissue orientation

Glutaraldehyde

- A. *Mechanism of Action*
  -Crosslinks proteins by forming methylene bridges between polypeptides at reactive side groups
  -Irreversible, added to protein, not coagulative



glutaraldehyde

Reaction with cell components

- -Preserves proteins & nucleoproteins excellently
- *slight reaction with lipids.*
- may denature enzymes & antigenic determinants due to rapid fixing properties.
- causes some cell shrinkage.

Type of glutaraldehyde

- Biological grade -less expensive & adequate for some studies, but may have impurities (glutaric acid) that alter the pH & osmolarity.
- EM grade (sealed under nitrogen in ampules) - best, most expensive. EM grade can polymerize if not stored properly. Should store at 4C in the dark. Polymerized glutaraldehyde lowers effective concentration.

Penetration

- 2.5 mm/ 24 hours at 4C
- 4.5 mm/ 24 hours at RT

Buffer

- -pH of cacodylate buffered glutaraldehyde may drop on storage
- -do not use above pH 7.5 - it polymerizes
- -phosphate and cacodylate buffer best; some organic buffers like PIPES and HEPES also work very well & in some cases better than phosphate and cacodylate.

Concentration

- Generally used at 2-6%, with 3% most common
- -If concentration too low (<1%) may fix too slowly and some cell components lost.

Temperature

- - Dependent upon structures of interest, works well from 4°C up to physiological temperatures.
- - Most often used cold or at RT

Tissue size

- -For immersion fixation, tissue piece should not be larger than 1 mm^3.

Osmolarity

- Usually used hypertonic (400-500 mOsm), <300 mOsm may cause swelling.
- Isotonic = 310 mOsm
- Hypotonic and isotonic solutions may cause some extraction, but some cells or tissues fix better.

Storage

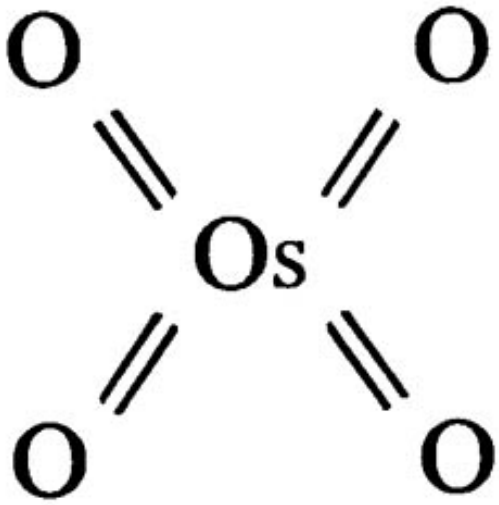
- Tissues stable up to 6 weeks @4°C - best results obtained when postfixation & embedding are done within 24 hrs.
- Prolonged storage may increase artifacts (membranous whorls) & extract components not cross-linked by the glutaraldehyde.
- 0.2M sucrose + buffer at 4°C may be used for prolonged storage

Safety

- Wear gloves, it is easy to develop a sensitivity & become allergic to glutaraldehyde.
- Use in fume hood

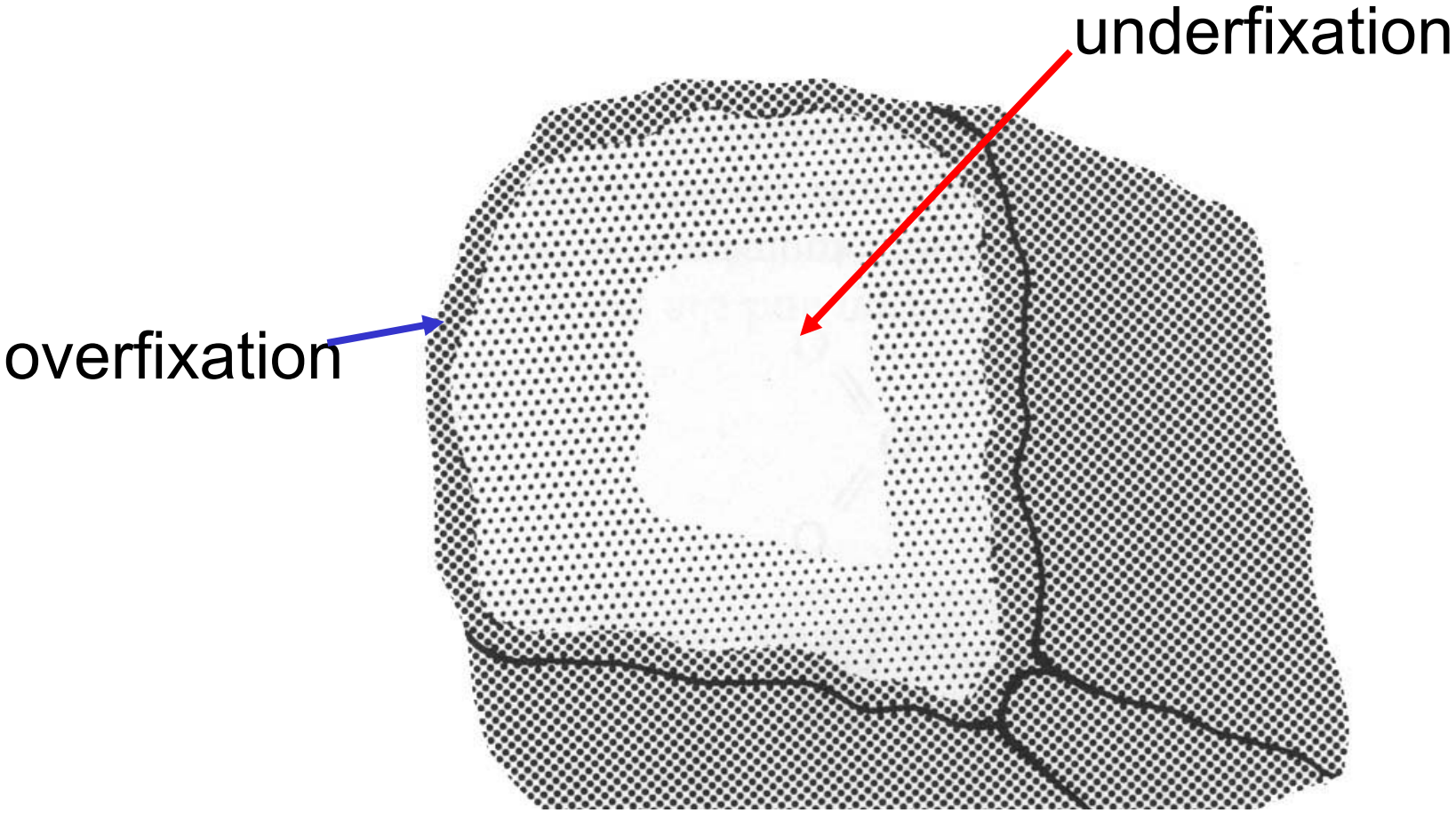
Osmium tetroxide

- Strong oxidizing agent, reacts primarily with lipids
- Not suitable for primary fix - penetrates & fixes slowly. Destroys membrane selective permeability
- Volatile, fumes may fix corneas, use in fume hood only.



Osmium tetroxide

- Penetrates slowly



Osmium tetroxide

- Used at 1-2% in buffer.
- Store in clean bottles & always use a new, clean pipette when removing some from bottle. Small amounts of contamination will oxidize, & the osmium solution ruined (turns violet or black).
- Neutralize using vegetable oil (corn oil)

Methods of Fixation

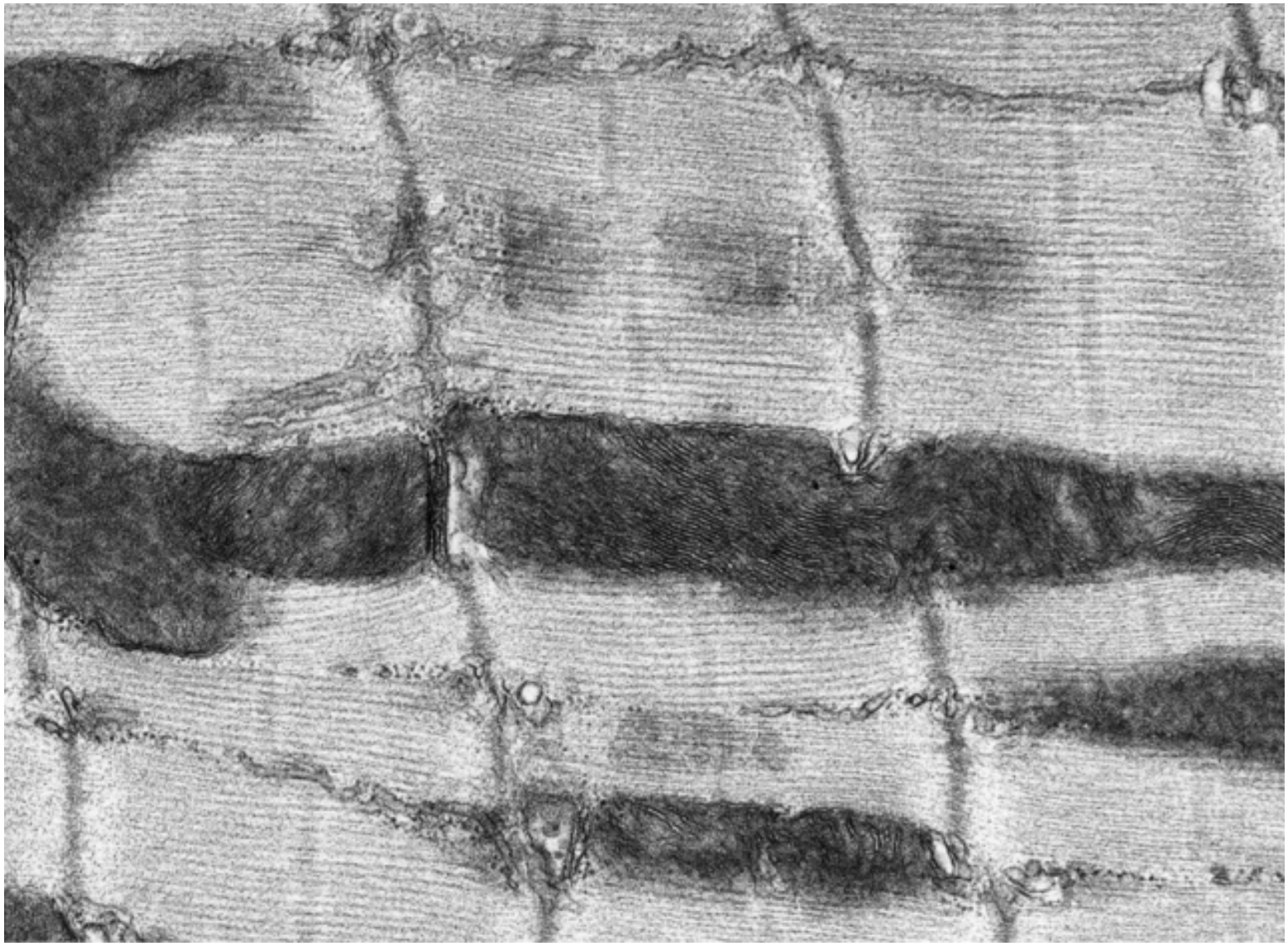
- **<u>Immersion</u>:** Rapidly remove tissue of interest from animal (euthanized or under anesthesia) or plant & mince tissue into 1mm^3 pieces while submerged in fixative. The rate of fixation may be increased via microwave irradiation.

Methods of Fixation

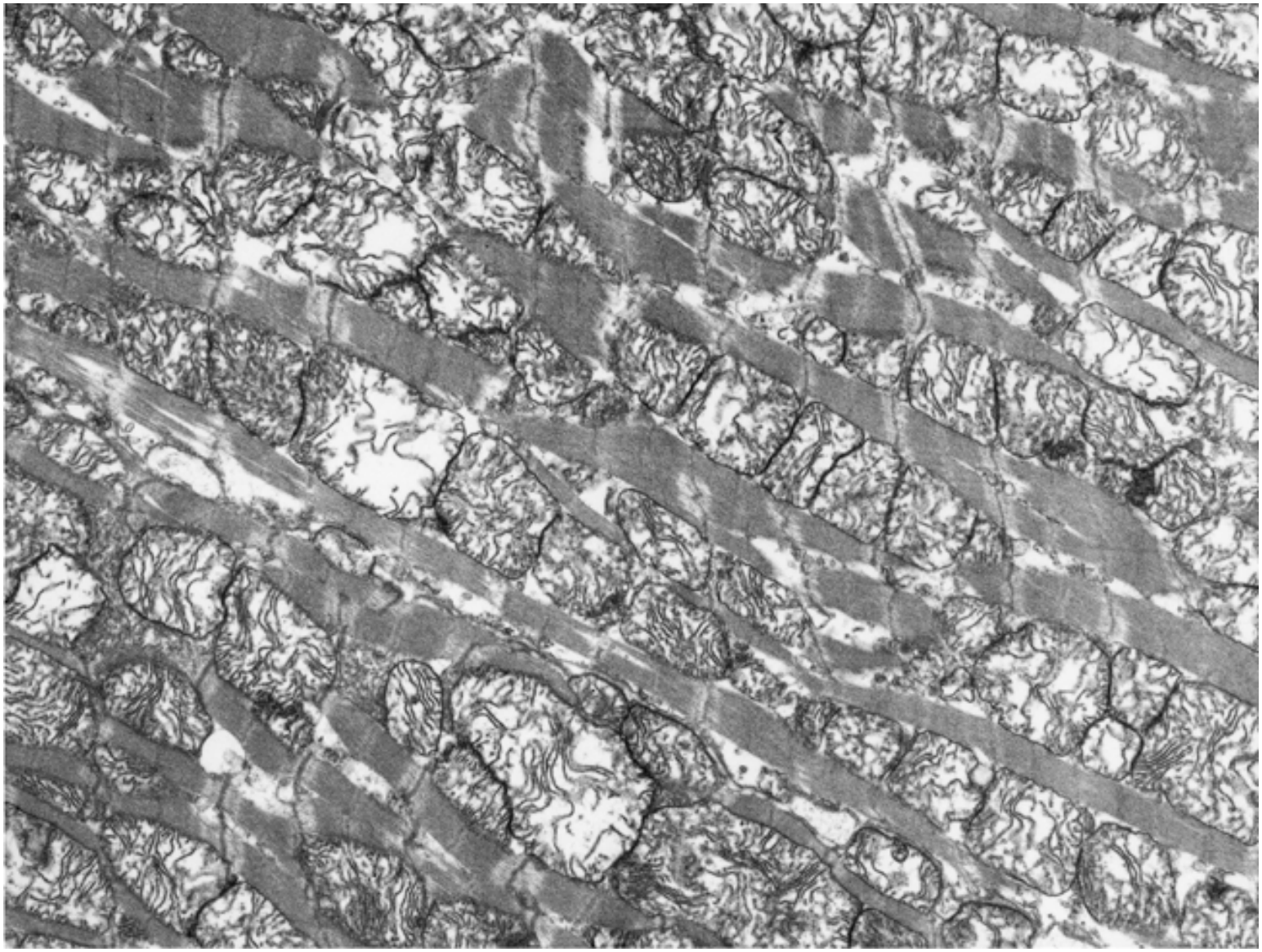
- **Perfusion:** Anesthetized animal has blood replaced by fixative. Needle or canula placed in the left ventricle or aorta (right ventricle cut to allow drainage) or blood supply to an organ may be canulated & perfused.
- Bolus of heparinized saline, Tyrode's or Ringer's solution (2-10 ml depending on animal size) prior to the fixative increases perfusion efficiency.
- Perfusion pressure should approximate normal blood pressure. For perfusion into heart or artery - fluid reservoir should be ~120-150 cm above the animal. For vein perfusion, should be ~20-30 cm above animal.
- After perfusion, tissues are removed & minced as per immersion fixation.

Methods of Fixation

- **Microwave fixation**: Microwaves in conjunction with immersion fixation produces excellent fixation. There is better preservation of antigens & less extraction of components from tissue.
- Tissues fixed by immersion & rapid bursts of low-energy microwave irradiation. See references for specifics.



Cardiac muscle - good fixation



Cardiac muscle - poor fixation

Specimen Preparation

Embedding

- aqueous or nonaqueous medium
- dehydration
- specimen orientation/types of specimen

Embedding

- Goal: Infiltrate tissue with liquid polymer (usually an epoxy resin) that can be hardened after infiltration.
- Polymer serves as a specimen support for sectioning.

Dehydration

- Goal: Remove water – allow infiltration on non-water soluble embedding medium
- Usually acetone or alcohol used.
  - gradient starting at 10-30% solvent (important if phosphate buffers used)
- Final changes of 100% solvent should be anhydrous.

Infiltration

Start with a mixture of resin & solvent in the ratio of 1 part resin to 3 parts solvent.

- resin concentration then increased in subsequent steps.
- time to infiltrate specimen – variable - depends on specimen block size & density.
- Infiltration time of ~ 24 hours adequate for most tissues.

Resins

- Epoxy resins - Poly/Bed 812 (Polysciences Inc., Warrington, PA).
  -can vary the hardness of epoxy based resins to match tissue characteristics.
- Acrylic resins – LR white
  -can embed from 70% alcohol

Water miscible resins

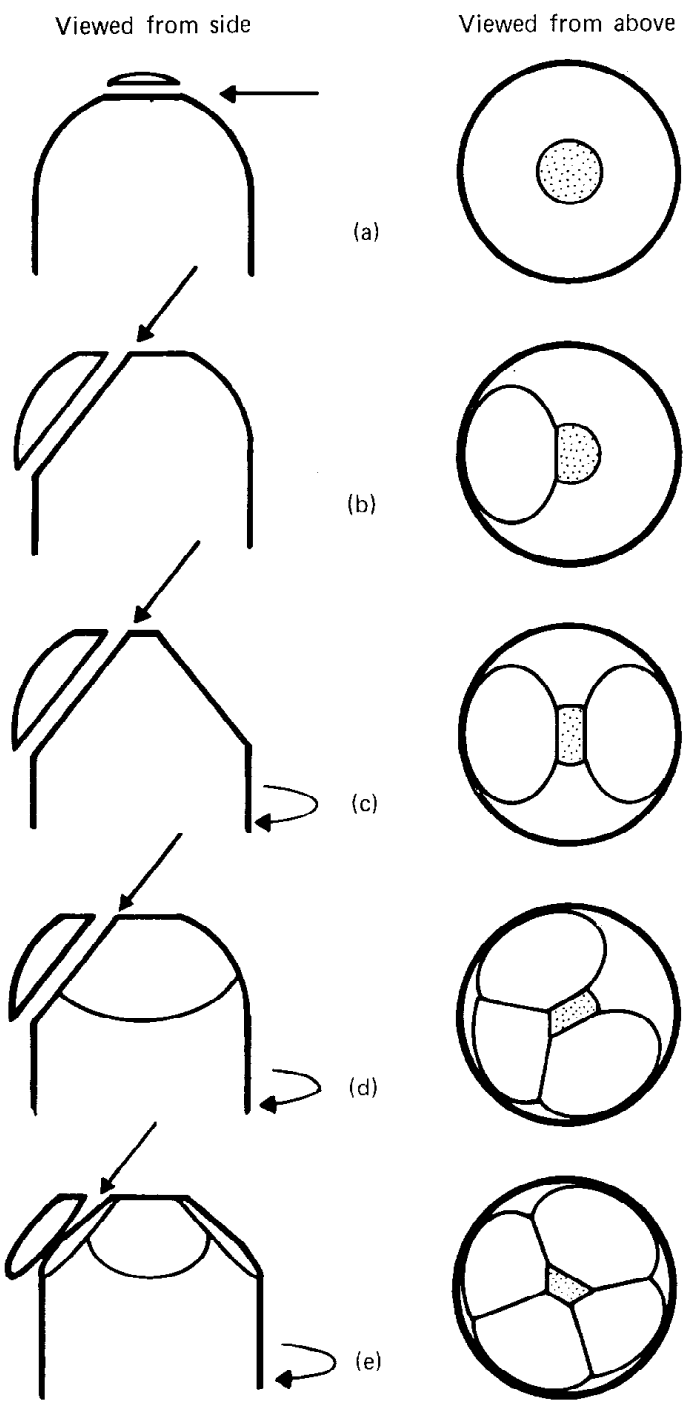
- Resins used to minimize extraction of lipids by dehydration solvents, or to preserve antigens of interest for immunocytochemistry.
  - Nanoplast - Melamine embedding resin
  - Quetol 651 -water soluble epoxy resin. Miscible with water, ETOH and acetone

Principles for resin handling

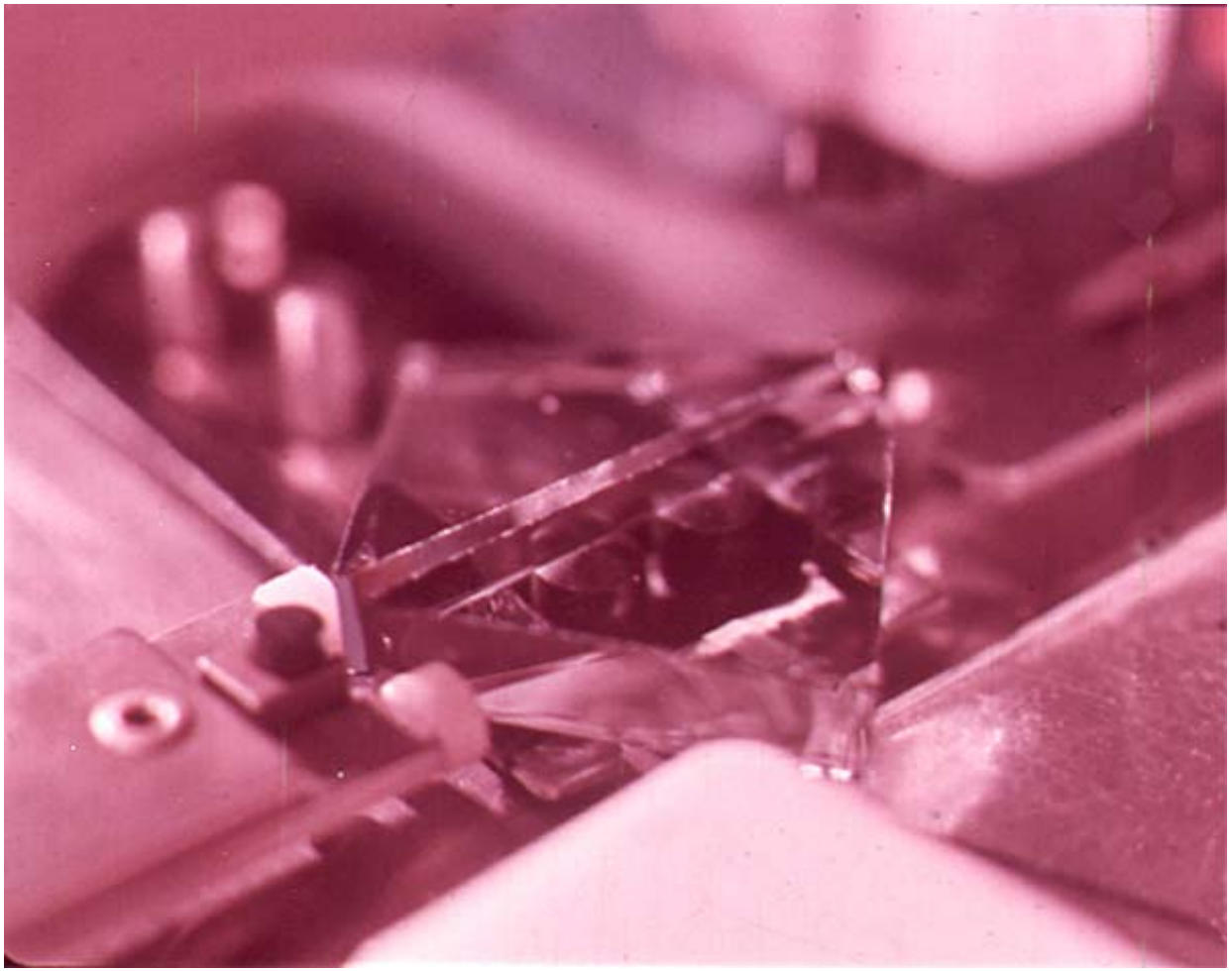
- **Safety:** Assume all components are toxic. Wear gloves, use in fume hood.
- **Mixing**: All resins should be mixed thoroughly before use. Incomplete mixing will result in poor blocks.
- **Disposal**: All unused resins should be polymerized before disposal. Old stock solutions that are not polymerized should be disposed of by institutional toxic waste control.

MICROTOMY

- Factors that influence sectioning
  - Tissue type: soft, hard, connective tissue, lipid, heterogeneity.
  - Tissue orientation
  - Embedding: soft, hard, brittle, resin type
  - Specimen block face
  - Cutting edge of glass knife

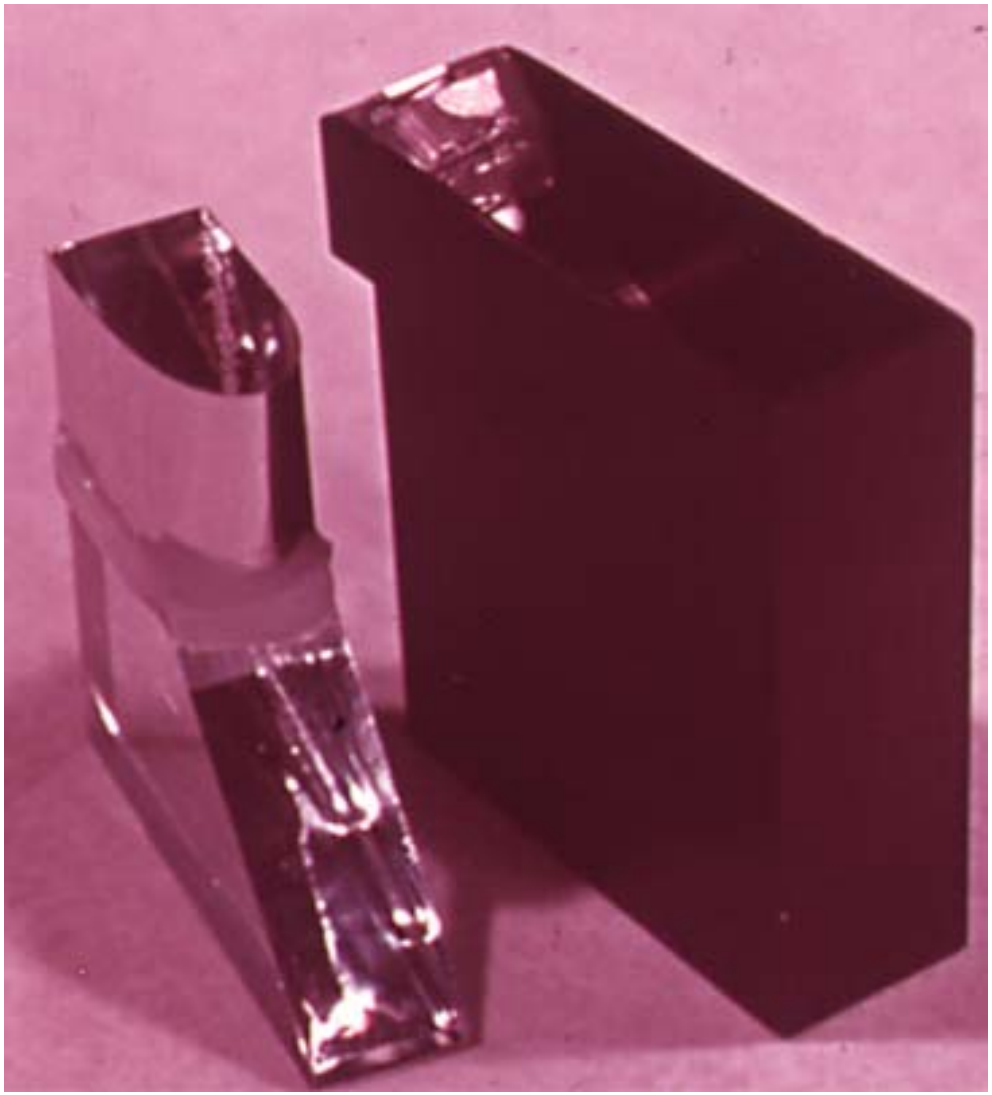


Tissue is trimmed to form a pyramid. The base of the pyramid is usually 0.5mm or less



Knives are made by scoring and breaking from glass strips

A “boat” is made on the glass knife with mylar tape and sealed with wax.

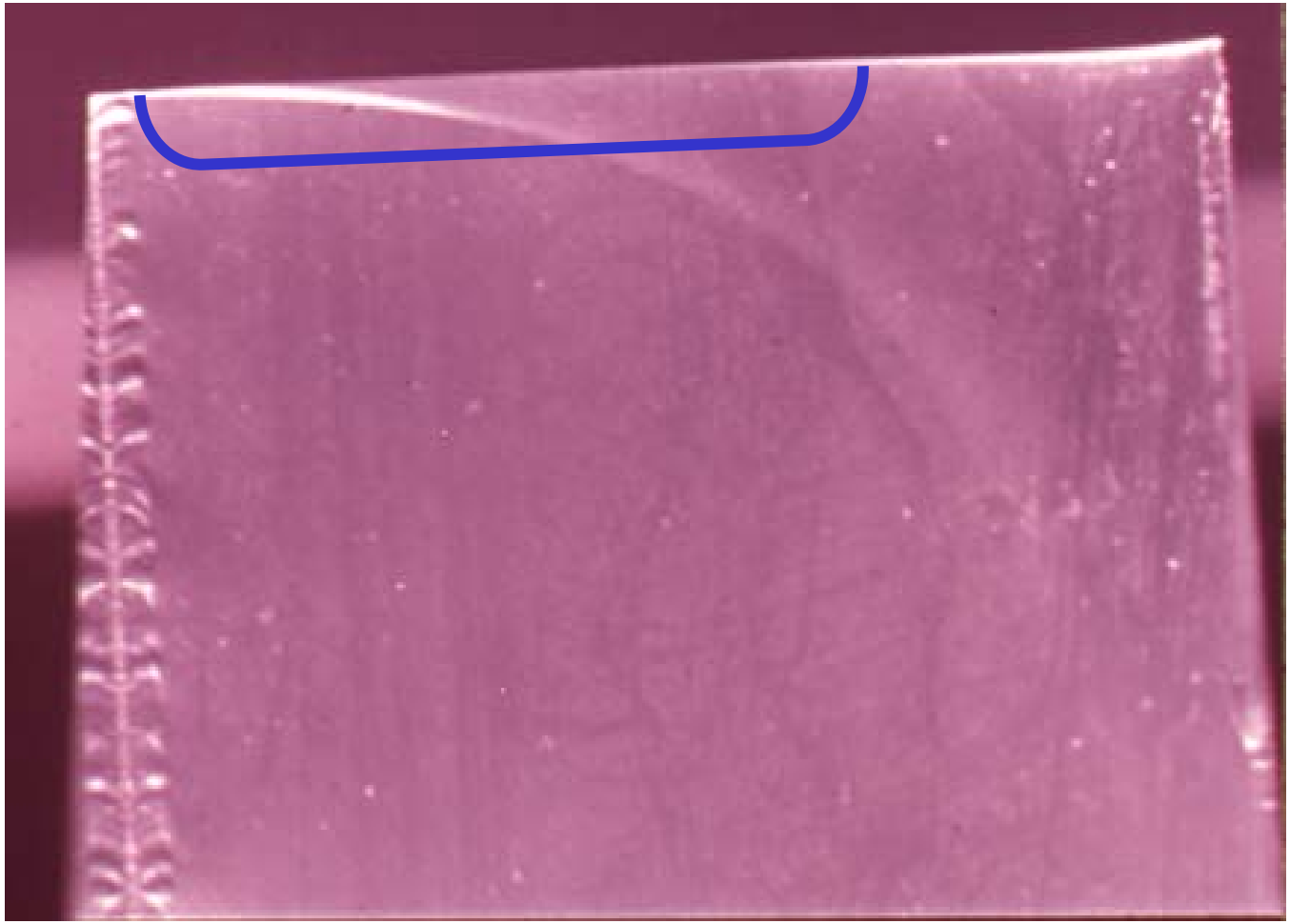


A disposable glass knife and a diamond knife

Cutting edge of glass knife

- Check knives under stereomicroscope, look at reflected light on edge.
- Stress mark runs to upper left hand corner of knife.
- Use knives which have straight edge or are slightly convex.
- Right side of knife may have saw-tooth appearance.

Usable edge of glass knife. The right edge may be used for gross trimming, but is unsuitable for thin sections



Knife angle

- Knives with a 45° angle are best & used most commonly
- The clearance angle between the knife and the block face may range from 1-6°, with 2-5° most common.
- The greater the clearance angle, the greater the stress on the knife edge.

Cutting speed

- The harder the block, the slower the cutting speed.
- Cutting too fast may cause localized heating, chatter, & damage the knife edge.
- For thick sections (1-5 mm/sec; 0.5-3 mm/sec for thin sections). Do not cut faster than 1 mm/sec on diamond knives.

Fluid in collecting trough

- Surface tension of fluid influences how sections float off after sectioning.
- Distilled water used most commonly. 10% EtOH or acetone may also be used if you need to decrease surface tension.

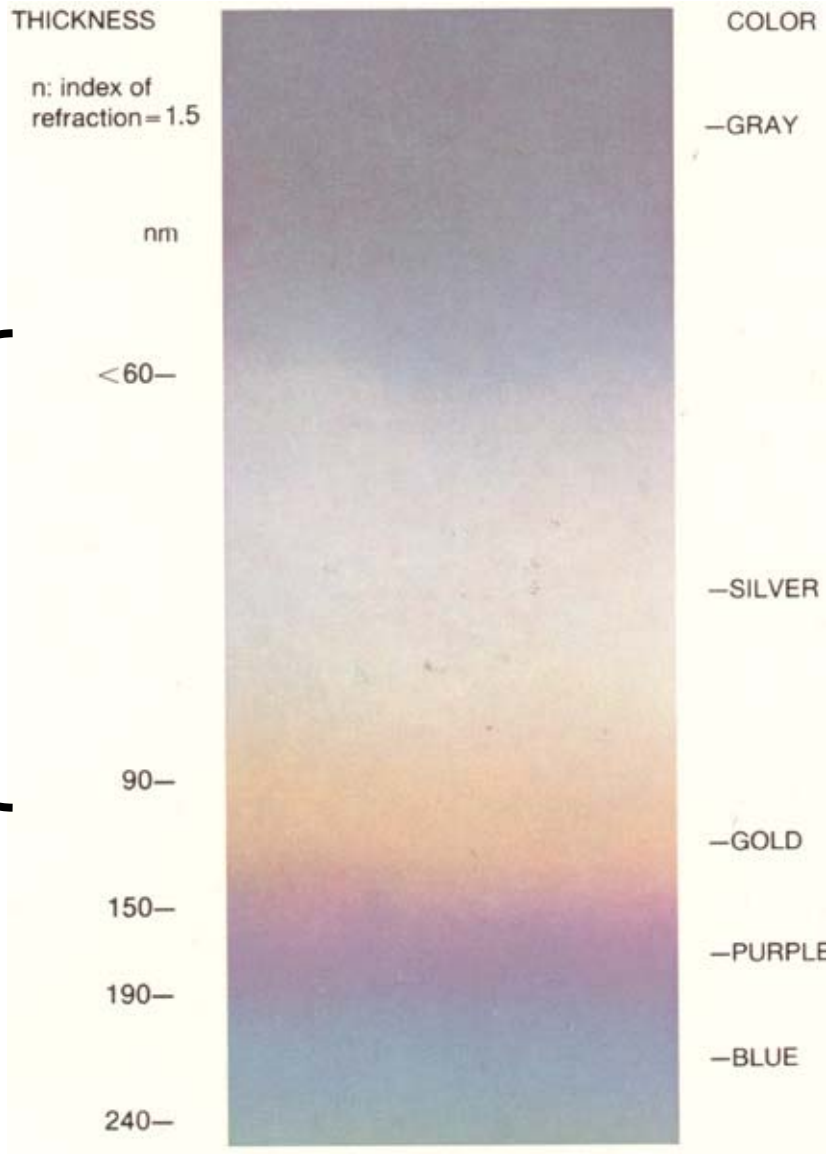
Thick Sections

- 0.5 to 2.0 μm in thickness
- Sections < 1 μm more difficult to stain. (Methylene blue/Azure II, Toluidine blue, Paragon, PAS-Iron Hematoxylin)
- Epoxy may be etched from sections with sodium ethoxide, & sections stained with any histological stain.

Thin sections

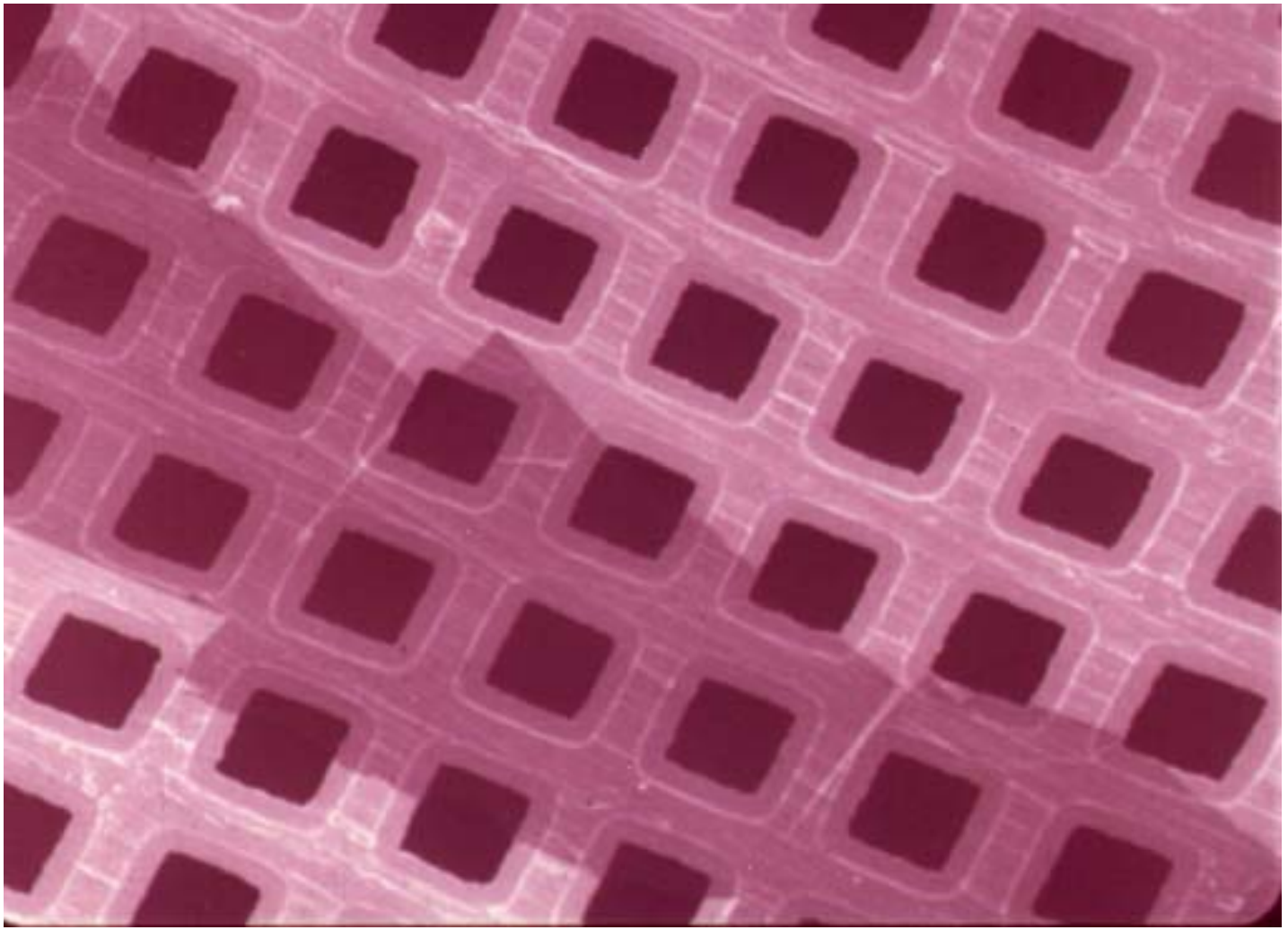
- 50 to 90 nm sections (pale silver to gold)
- The thinner the section, the > resolution, but thinner sections have less contrast.
- For low magnification work, pale gold to gold sections give better contrast.
- Staining quality depends upon tissue type, embedding medium, section thickness, & buffers used in fixation.

Usable thickness for 80-100 kV



Cut sections float off on the water

Sections are picked up by touching a grid to the sections



Sections mounted on a grid. Note that the section only covers ~ 4 complete grid squares.

Staining Tissues/Sections

- Stain sections soon after sectioning & not too far in advance of when they will be photographed.
- Osmium tetroxide is a stain. Contrasts lipids.

Staining Tissues/Sections

- Most common method is to stain the tissue after sectioning using uranyl acetate & Reynolds' lead citrate.
- uranyl acetate (saturated aq) may be mixed at various ratios up to 1:1 with absolute ethanol or methanol or a saturated aqueous solution may be used. Staining times range from 10 to 40 min.

Staining Tissues/Sections

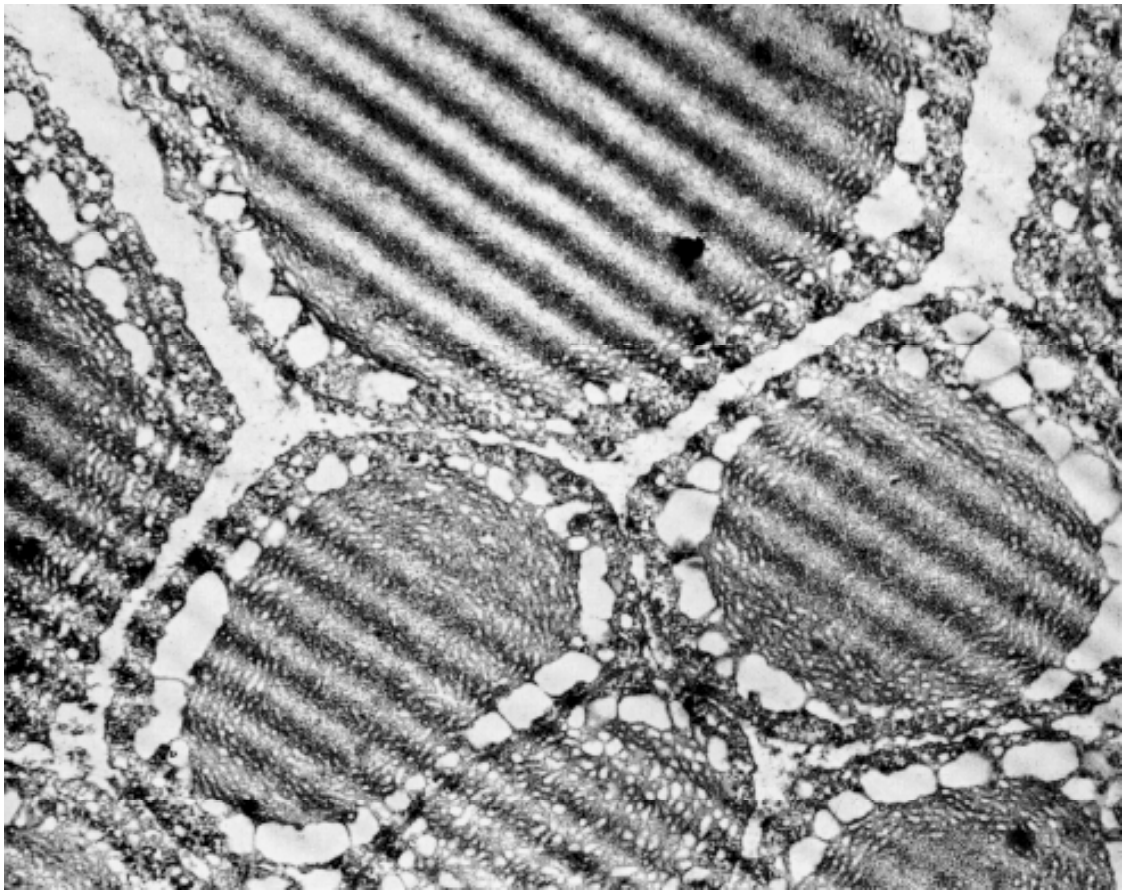
- Reynolds lead citrate used following UA staining.
- Exposure to CO_2 in air or dissolved in washing solutions results in lead carbonate formation (not good). Wash water should be boiled to drive off dissolved CO_2 & staining performed in a petri dish (it helps to have some sodium hydroxide pellets in the dish to absorb CO_2. Stain 1-4 min.

Sectioning problems

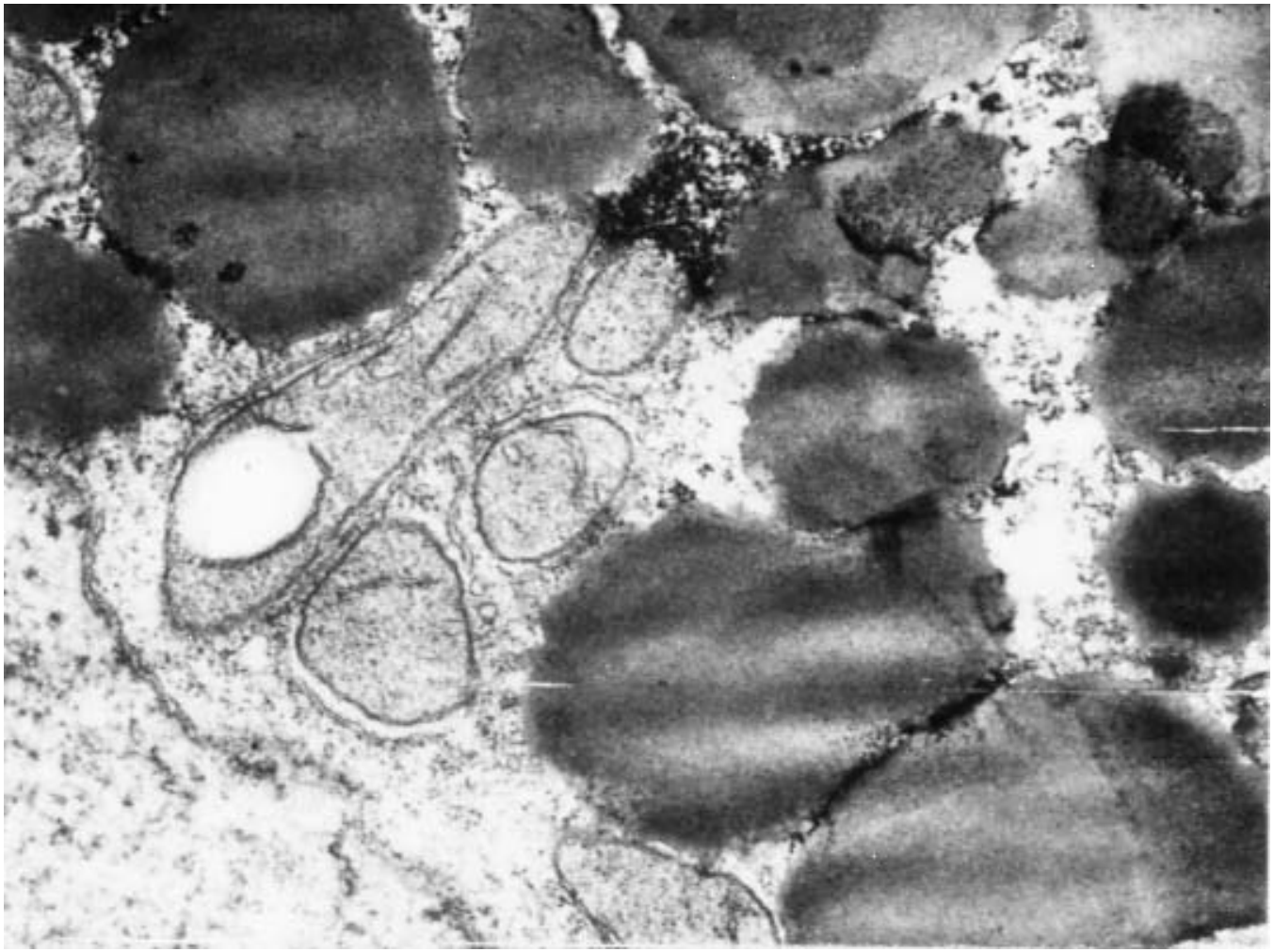
- **Chatter**: harmonic vibrations in building, instrument, or set up in specimen block by striking of block to knife edge. Variation in section thickness, lines run parallel to knife edge.
- **Compression**: Section height < block face height. Occurs to some degree in all sections, but incorrect knife clearance angle, or dull knife may exacerbate. May be cured to some degree by flattening with solvent vapors or heat. Sections may have dull or milky appearance.

Sectioning problems

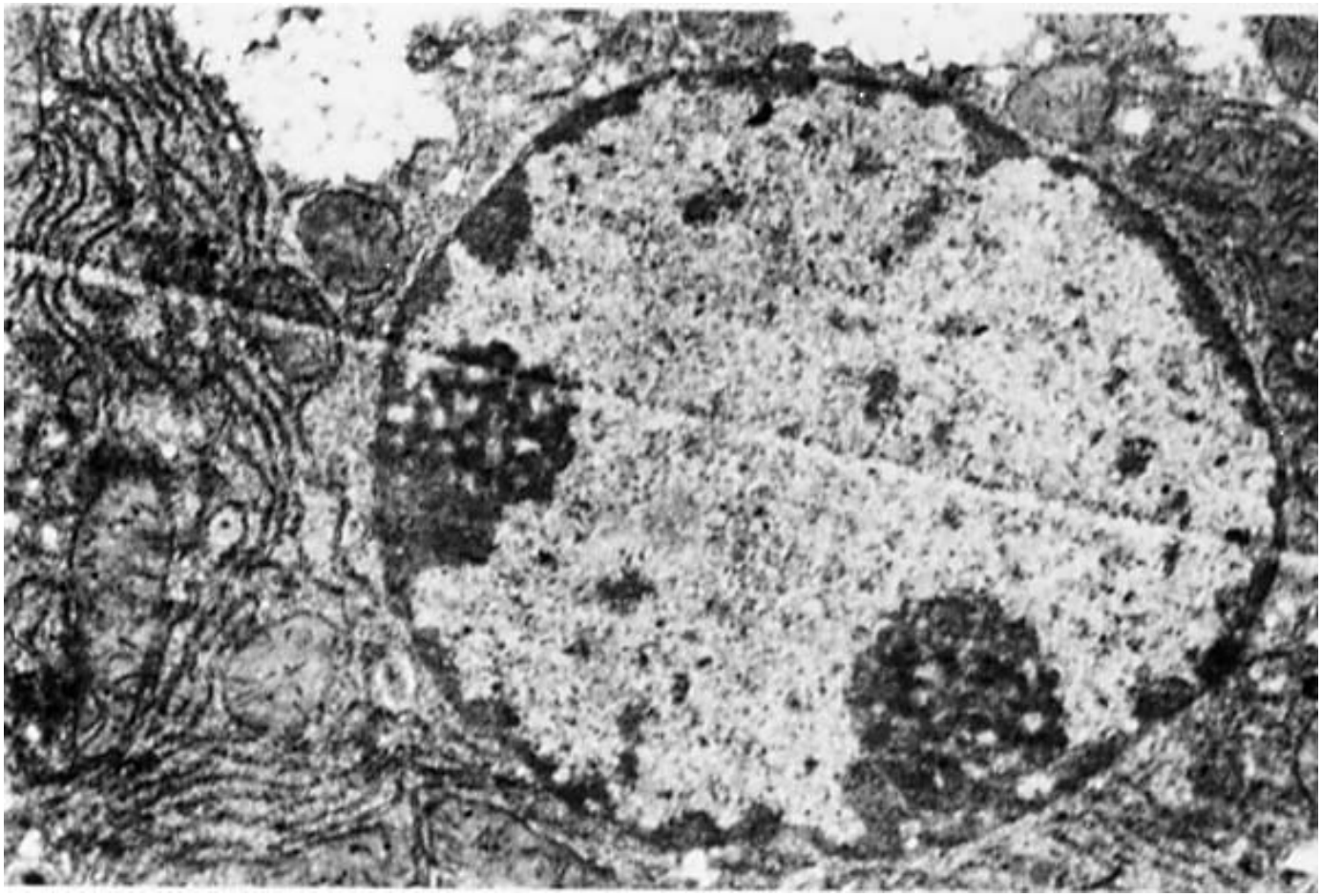
- **Knife marks**: Defects in knife edge. Run perpendicular to knife edge.



CHATTER



Compression



Knife mark