P40 Technique

SHEET PLASTINATION

I.- SPECIMENS PREPARATION AND SLICING:

I.1.- Brain.

- Fixation is necessary to yield the brain firm enough for slicing
- **Types of fixation:**
  - **Perfusion**, is the ideal method to achieve the best fixation
  - **Immersion**
  - **Injection**
- **Fixative Solutions:**
  - Formalin fixation 10%
  - Specimens that have been fixed using formalin containing other chemical additives should be used cautiously as the additives may cause adverse reactions with the resin
- **Brain may be embedded in 20% gelatin** to keep the various pieces of the slice together as a single unit **(optional)**
- **Slicing:** The proposed plane of section of the brain is determined and the brain is cut into two pieces with a brain (large) knife.

- Deli/Meat slicer. Slices are prepared with the slicer at a determined thickness. Slice thicknesses of 4, 6 or 8mm are preferred since standard gasket sizes for these thicknesses are available.
    - Clean the surface of the slicer disc with water after each slice
- A produced slice is placed on a filter paper and then onto a metal or plastic (acetone resistant) grid.

- **Storage:** 5 - 10% formalin as long as needed.
- **Rinse:** Tap water, overnight to rinse the formalin from the slices.
- **Pre-cool slices** to +5^{o}C, prior to dehydration

- **I.2.- Body slices:** specimen is positioned for anatomical clarity and frozen preferably in an ultra-cold deep freezer
    - **Fixation:** Preferably none. Fresh tissue is preferred although; tissue should be fixed in formalin if deemed appropriate to decrease the potential for exposure to biohazards that may be associated with routine handling and sawing of biological tissues. Preservation of tissue color is the best reason not to use formalin.
    - Specimens covered with hair should have their hair clipped.
    - Formalin containing other chemical additives should be thoroughly rinsed to deplete the tissue of any chemicals that may react adversely with the resin.
    - **Freezing of specimen:** Whole cadavers or large specimens should be cut into smaller more manageable

portions. This not only facilitates handling of the specimen but also prevents excessive thawing of the remaining specimen while sawing is being done.

- Ultra-cold freezing is necessary to yield the best tissue slices. 7-10 days at -70ºC

- **Slices:** 2 - 4mm
  - Band saw :The slices of the frozen specimen are cut on a band saw.
    - The guide stop is set for the desired specimen thickness (2-3mm) and serial sections are sawed.
    - Use dry snow or liquid nitrogen to keep the guide stop frozen during slicing.
  - Submerse the slices in cold acetone -25ºC
  - Dust removal**:**
    - Submerging the frozen slice in the first dehydration bath and scraping the dust from the specimen while submerged, or
    - Flush with stream of running tap water, quickly to prevent thawing prior to submersion in cold acetone
  - Place the slices on aluminium grids: grids with their cleaned slices are stacked in the cold acetone (-25°C). They must be submerged in the acetone without option to float. The stacked grids with their slices may be tied together in bundles or placed into

a basket for convenience and ease of transfer from one acetone bath to another or into the impregnation resin.

- **Storage:** in freezer -25ºC

II.- DEHYDRATION: The recommended dehydration procedure for plastination is freeze substitution in -20 to -25°C acetone. Shrinkage is minimal when cold acetone is used. Ethanol dehydration promotes excess shrinkage of brain tissue and should not be used. For all plastination techniques, acetone is ideal and serves both as the dehydration agent and defatting, as well as the intermediary solvent. Also, acetone readily mixes with the resins used for plastination.

- **Temperature:** -20°C Acetone
- **Duration**: 2 - 3 acetone baths
- Approximate acetone to specimen ratio: 10:1. An estimate of acetone volume for one human brain is 25l.
- Slowly lower slices into cold acetone while slowly moving back and forth: this exposes the slices to continual cold acetone and releases trapped air bubbles.
- **Dehydration protocol:** After two or three days in each acetone bath, the purity is checked with an acetonometer after stirring the acetone bath. The acetone temperature must match the temperature calibration of the acetonometer. Most acetonometers are calibrated at +15°C, +20°C, or -10°C. Therefore acetone must be

warmed or cooled to match the calibrated temperature before measuring and recording the purity reading

  - **Bath 1:** Acetone 90% 4 days, 2/3 of time. This first acetone bath may be less than 100% (<90%). The slices should be tilted a few degrees as they enter the acetone bath and agitated after submersion to remove trapped air bubbles.
  - **Bath 2:** Acetone 100% 2 days, 1/3 of time. After 2 to 3 days, the basket of slices is moved to the **second** (new) 100% cold acetone bath of a similar volume
  - **Bath 3:** Acetone 100%. If the acetone concentration is more than 98%, dehydration is considered complete. However if more than 2% water remains, a **third** dehydration bath must be used to complete dehydration
  - **Final** Acetone **reading** at least **98%**
- Move (agitate) acetone containing the slices twice a day - Hastens dehydration
- **Caution:** Dehydrated slices, especially brain, become brittle and break easy - Handle with CARE!
- **Degreasing of body slices** is accomplished by setting the dehydrated slices out of the deep freezer into room temperature for the necessary time to **degrease** (1 - 2 weeks). Monitor degree of degreasing by observing fat

color and acetone color. Acetone will turn yellow as the fat is leached. The acetone should be changed to a fresh bath when the yellow color has become intense. Fat will change from its white color to opaque when defatting is nearing completion. If more transparency of fat is desired, dehydrated slices may be placed into methylene chloride (dichloromethane) for one or two days. Monitor degreasing in methylene chloride daily. When slices are appropriately degreased, transfer the slices from their bath (acetone or methylene chloride) into the impregnation resin

---

III.- IMPREGNATION

Impregnation: P 40 resin

Forced impregnation, the process of drawing a solvent (i.e. acetone that replaced the cellular fluid during dehydration) out from the cellular structure of the specimen and replaces it with resin (P40).

- **Immersion into P40 resin:**
  - The dehydrated brain or body slices are transferred into and submerged in a room temperature impregnation bath. This bath may consist of P40 resin alone or may be a combination of P40 resin plus 1-2% A4 (activator).
  - Place cold sections into **cold P40:** 1 day prior to impregnation. it is a beneficial to allow the slices to sit

in the P40 resin overnight and equilibrate before applying vacuum and commencing forced impregnation

- Keep covered to prevent light exposure: **UV light is the catalyst for P 40!**

- **Forced Impregnation:**
  - Place resin with slices in vacuum chamber,
  - 2cm of resin above the slice level
  - Cover glass vacuum chamber top with black: block out UV light

- **Vacuum:** The vacuum pump is turned on and allowed to warm to working temperature. Once the pump has warmed, the slices in the impregnation resin are placed in the vacuum chamber at the room temperature and vacuum is applied. The vacuum chamber must be kept darkened

- As the pressure is lowered, the acetone (because of its high vapor pressure) boils out of the slices leaving a tissue void into which the polyester resin enters. Frequent regulation of the rate of evacuation is necessary to keep the impregnation boiling rate active

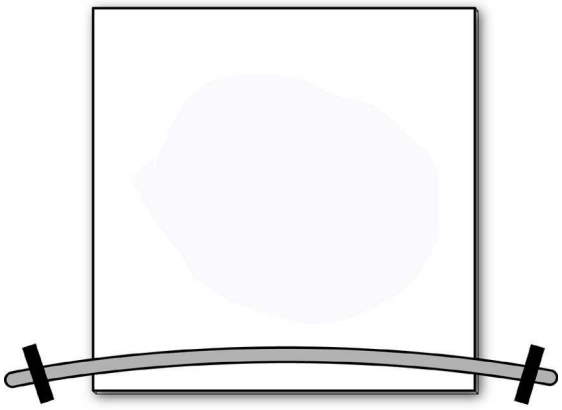
- **Vacuum** : Commence and adjust so that many bubbles rise slowly
  - Air intake valve :
    - Between pump and the impregnation chamber
    - Close to increase vacuum/lower pressure.

- Decrease pressure (Increase vacuum) hourly **if bubble production has decreased**.
- **Final vacuum:**
    - At room temperature (15^{o}C) not below 9 mm Hg or **at least 10 mmHg**.
    - If impregnation is in a cold room (4^{o}C): Final vacuum - not below 5 mm Hg
    - If lower pressure is reached, monomeric styrene extraction will commence which will clog the pump.
- **Impregnation complete** when**:** Appropriate final vacuum (9 mm) and acetone bubble production is decreased and larger bubbles predominate

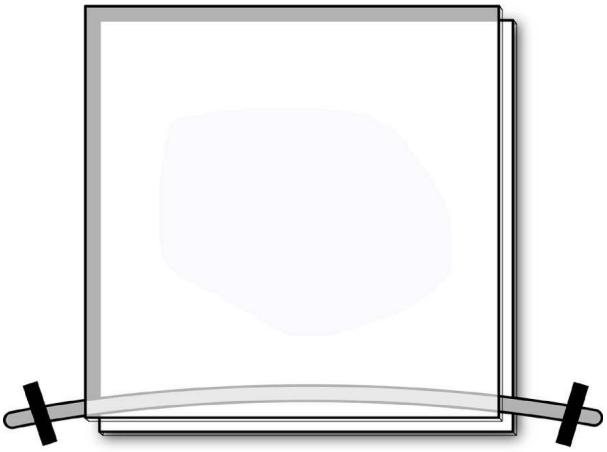
- **Impregnation time:** 24-48 h
- Store in the impregnation polymer in refrigerator or freezer until ready to cast.
- Polymer used for immersion and impregnation can be reused, Keep the container covered to minimize light exposure.

IV.- CASTING Y POLIMERIZATION

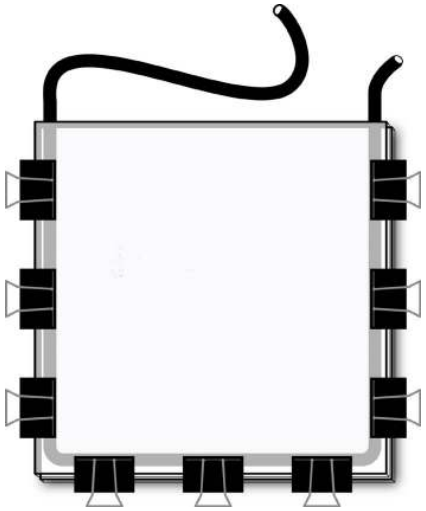
- **Casting Slices in flat chambers:**
    - **Casting Slices in flat chambers**
    - **Glass characteristics:** 2mm regular or templated glass: Choose size at least 3 inches (7 cm) wider than the largest slice.
        - Thinner wall (2 mm) of the glass better dissipation of heat which is produced during curing,
        - Thinner wall of the glass decreases curing time.
    - **Gasket to place between the glasses:**
        - **Silicone tubing:** Need fewer clamps
        - **Silicone gasket, PVC gasket or tubing:** Need more clamps to seal the chamber.
    - **Assembly of flat chamber:**
        - **Place bottom plate on glass platform -**
        - Cut tubing to length of perimeter of glass,
        - Place silicone tubing across bottom edge (up 2 cm from bottom) 2/3 of tubing on one side and 1/3 on other side. The longer end will be used to close the top after filling



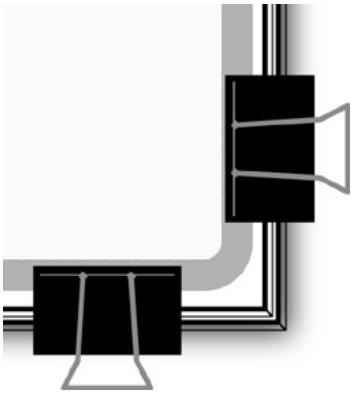
- **Place top plate on:**
- 1 clamp - 2 cm from each bottom corner,
- Clamp directly over the tubing,



- Turn tubing up both sides and position 2 cm from perimeter: Keep a sharp bend (90º) at corner,
- Place 2 side clamps on each side over the gasket,
- Fold-back clamps are placed along the perimeter of the bottom glass over-lying the gasket. Add clamps as needed to sides and bottom

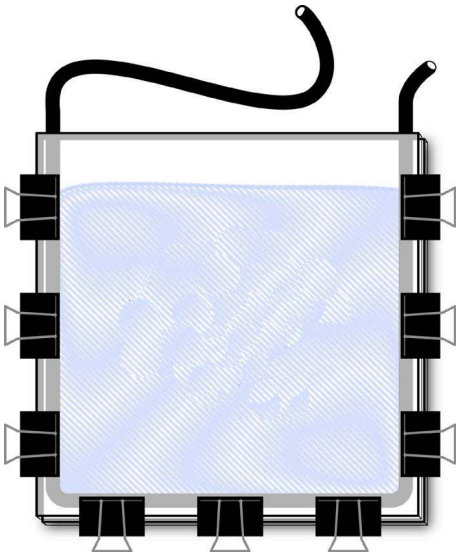


- **Center** of clamp pressure point to be on center of tubing.
- Clamps are positioned directly over the gasket. The top is left open with the excess gasket hanging to each side. The clamps secure and seal the glass plates with the gasket along the bottom and the sides

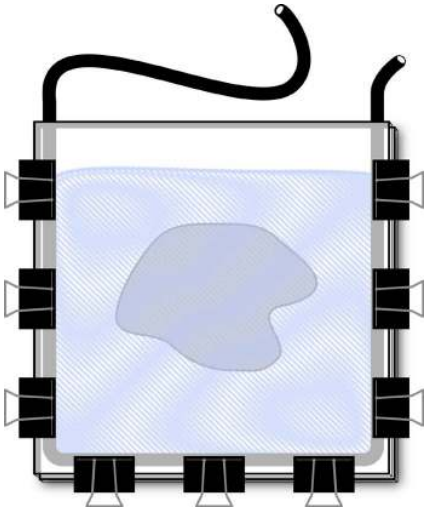


- **Filling flat chamber with P 40 resin:**
  - Resin, slices & chambers - should be similar temperature

- To prevent slices form rising (if slice is colder than resin, it will rise),
- Fill chamber ¾ full with fresh P40 or well-mixed P40/A4 mixture (100:1-2).

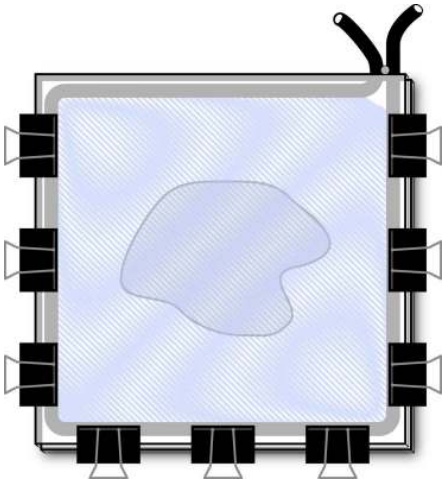


- **Put slice into the flat chamber:** The slice is inserted through the top opening and the flat (casting) chamber
  - Use spatula to lift slice,
  - Slide into chamber.



- **Extract air bubbles:**
    - Sit chamber vertical or at a slight angle; many bubbles will rise
- **Trapped bubbles:** Insert 1 mm wire to tease bubble out,
    - Incline to a more horizontal position to allow bubbles to rise,
    - Insert wooden wedge between top glass openings.
- **Center slice in chamber**: Insert 1 mm wire with hooked end and center the slice
- **Sealed method:**
    - Fill chamber with polymer to with in 2 cm of top of chamber,
    - **Pour in resin near edges** (rather than center) to fill the chamber, it creates currents that raise bubbles
    - Push long end of tubing in between plates,

- Where long end meets short side tubing - seal with Biodur sealant.



- **Extract air bubbles from sealed chamber:**
  - Angle such that air bubbles pool in 1 corner
  - Insert 1 mm wire or 18ga hypodermic needle between gasket and glass to allow air to escape.
- **Position slice:**
  - If sealed: Insert one or two (2 - 4 mm) stainless steel ball bearings before top gasket is sealed,
  - Using a strong magnet, push the slice into an appropriate position,

UVA light curing:

- **Electric UVA light:**
  - Two 40 watt UVA Lights (black lights) above and below, at a distance of 15 cm from the flat chamber. A minimum of one hour is recommended for curing,

depending on the wattage and distance of the UVA lamps.

- **Position of the chamber:**
    - **Horizontal:** Prevent brain from sinking down onto bottom tubing
- **Maximum Temperature** of curing slice: **35°C** : During light exposure, it is necessary to cool the chambers on both sides either by ventilators or by blowing compressed air over both sides of the glass chamber. Excess heat may cause reddish orange spots especially in gray matter or dense tissues.
    - **Use fan/ventilator** or compressed air blowing across the chambers to cool,
    - Thicker slices (6-8 mm) more heat produced.
    - Every 25 minutes, turn off UV light to allow unit to cool and in 25 minutes expose again for 15 -25 minutes.
- **Two Stages of curing:**
    - Jellification - loss of transparency after 30 min,
    - Hardening - sharp differentiation of gray and white matter.
- **Bulging of flat chamber:**

- **Prevent bulging:**
    - Lay flat chamber on another glass plate after curing
    - Dirt specks will show up as shadows.
    - Metal close to the chambers will cause fogging of the polyester.

Dismantling of flat chambers:

- 24 hours - Remove from UV lights or day light:
- Remove clamps,
- Remove tubing &
- If glass does not separate from cured slice: With a pointed scalpel, scrape the junction of the cured resin and the top & bottom glasses,
- Or let stand 2 - 3 hours - Gently pull apart
- **Cover with foil** (plastic wrap) to keep the sticky edges from making a mess.
- **Saw:** Edges off with foil in place, to prevent uncured polymer of the edge from making a mess**.**
- Clearing of glasses and gasket tubes:
    - Soak in: Acetone; Enzyme detergent, bleach and hot water; or put in dark bag
    - Clean both sides of glass
    - No scratches: Imprint will be on sheet

Storing P40: -20^{o}C: 5 year+ shelf life

- +5°C: 1 year shelf life
- Room temperature: 6 months.

Before use: turn container 10 times to reunite separated components

PROTOCOL SUMMARY

1. Protocol Summary for P40 - Polyester Slices:

Day 1: Slice, rinse and cool slices

Day 2: Immerse in first acetone bath (<90%)

Day 5: Immerse in second acetone bath (100%)

Day 7: Immerse in third acetone bath (100%, If needed)

Day 9: Immerse in P40

Day 10: Impregnate with P40

Day 11: Cast and UVA light cure

Day 12: Open flat chambers, cover with plastic wrap, saw and sand

2. Materials:

2 mm glass - Easier dissipation of heat (standard 35 x 45 cm)

Conversion: inch to metric:

1/16" = 1.6 mm

3/32" = 2.4 mm

1/8" = 3.2 mm

3/16" = 4.8 mm

Tubing (fewer clamps)

Gasket (more clamps)

Clamps

3. Curing:

a. Near Horizontal

b. Catalyst - UVA light

1'. Shadow not direct sunlight

2'. UVA lights above and below

a'. UVA lights: 25 cm

3'. Mercury vapour

b. Geothermic reaction - polymerization

1'. <+35^{o}C

2'. To help avoid Red/orange - spots in brain cortex

a'. Increase light - chamber distance

b'. Cooling system

(1) Fans (ventilator)

(2) Cool room

(3) Thinner slices

4. Safety:

1. Good ventilation
2. Gloves
3. Goggles - when filling chambers
4. Sun glasses with UVA
5. Acetone spark precautions
6. Wash P40 off skin with cold water and soap

5. Pump speed:

1.5 m^3 = 4.5 Ft^3

3.0 m^3 = 9.0 Ft^3