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Silicone Plastination of Biological Tissue: Cold-temperature Technique Biodur™ S10/S15 Technique and Products

K. DEJONG[1*] and R.W. HENRY[2]

[1]*Department of Anatomy and Embryology, Academic Medical Center, Amsterdam, The Netherlands, Europe.*

[2]*Department of Comparative Medicine, College of Veterinary Medicine, University of Tennessee, Knoxville, TN, USA.*

[*]*Correspondence to: Telephone:* (31) 20 566 9111; *Fax:* (31) 20 697 6177; *E-mail:* k.h.deJong@amc.uva.nl

Abstract: Since its development nearly thirty years ago, S10 silicone plastination has become the gold standard for preservation of biological tissues. Silicone plastination is the most versatile plastination methodology as it may be used for whole cadavers or organs and portions or slices thereof. The Biodur™/German/vonHagens'/cold-temperature technique yields the most exquisite specimens with accurate surface detail and is the classic method of plastination. Ambient temperature silicone plastination yields similar results as cold plastination and will also be described.

Key words: **plastination; silicone; S10; S15; S6; S3**

Introduction

The goal of plastination is to replace tissue fluid with a curable polymer. Most often silicone, polyester or epoxy is used (von Hagens, 1979a; 1979b; 1986; von Hagens et al., 1987; Bickley et al., 1987; Henry and Nel, 1993). Once the polymer is inside the specimen (cells and interstitium), the polymer is cured (hardened) to keep the silicone in the specimen and to make the specimen dry.

Chemicals used in silicone-plastination include:

- Acetone and possibly methylene chloride
- Silicone polymer
- Catalyst to prepare silicone molecules for elongation and cross-linkage
- Chain extender to promote the formation of longer chains of silicone molecules
- Cross-linker to form a 3-D meshwork of elongated silicone molecules by side to side linkage

The general steps of Biodur™ silicone plastination are described in addition a listing of the specific details of Biodur™ S10 silicone-plastination. These basic tenants of plastination are also applicable to the generic plastination polymers. This basic protocol may be modified to utilize the generic silicone products and other methodologies.

Plastination consists of the following steps:

1. **Specimen preparation (dissection and fixation)**
2. **Dehydration**
3. **Defatting (degreasing)**
4. **Impregnation**
5. **Curing**

With silicone plastination, fresh or embalmed (formalin-fixed) specimens may be used. In general, using fresh tissue may yield a more flexible and pristine specimen than if specimens are embalmed or stored for

a long time in formalin. Selected specimens may or may not be fixed with formalin. However, tissue may be fixed to prevent any potential biohazard risk associated with the handling of human tissue (Ulmer, 1994; Smith and Holladay, 2001).

In substituting the solvent (acetone) for the silicone polymer-mix, a mixture of silicone polymer (S10 or S15) and catalyst with chain extender (S3) is prepared and referred to as the reaction-mixture or impregnation-mixture. Mixing the silicone polymer with the catalyst and chain extender starts the reaction of elongating the silicone molecules, thus over time silicone chain length increases which results in a more viscous impregnation reaction-mixture. Chain elongation is retarded at temperatures lower than -15°C. Therefore, a deep freezer at -15°C is used when impregnating with the reaction-mixture or when storing the reaction-mixture. A temperature of -25°C or lower is best.

The Biodur™ products for silicone plastination include:

S10: Silicone polymer
S15: Silicone polymer of lower viscosity than S10
S3: Catalyst and chain extender (pre-mixed)
S6: Cross-linker

Materials and methods

The basic steps of silicone plastination are specimen preparation, dehydration, degreasing, impregnation and curing.

Specimen preparation

Plan and Dissect: The specimen should be planned before starting the project. Consider how the final specimen should look and proceed with dissection to carry out the plan (Henry et al., 1997). The plan may be modified as needed or desired. If vessels are to be highlighted by filling the vascular system, injection should be done prior to dissection and sufficient time allowed for the injection material to solidify, since vessels are often damaged and leak during dissection. Keep the desired structures intact but remove excess fat and/or connective tissue. Begin as soon as practical to position (form) the specimen into the shape/position you desire for its final presentation. Hollow organs (chambers of the heart, airways, gastrointestinal and urogenital organs) should be flushed with running tap water to remove contents and cleanse the interior of the specimen. Appropriate sized cannulas or tubing, inserted via natural ports and secured in position, will aid and allow efficient flushing and dilation. Dilate gastrointestinal organs to the desired volume with water (Fig.1). As well, hydrostatically dilate hearts to over come systole and rigor (Tiedemann and von Hagens, 1982; Henry, 1987; Oostrom, 1987a).

Fixation: Fixation is desirable, but not essential (Oostrom, 1987b; Smith and Holladay, 2001). Formalin with no additives is best. To maintain the shape of thin-walled hollow structures and assure proper dilation during curing, it is beneficial to fix such specimens in their desired contour and dilated volume with 10% formalin. The best heart preparations must be hydrostatically dilated and then fixed by dilation (Henry, 1987; Oostrom, 1987b). Specimens may also be prepared without fixation. Eliminating the fixation step in some instances may yield a more flexible specimen. Specimens which autolyze rapidly (pancreas, brain) or are contaminated with pathogens should always be fixed (Riepertinger, 1988; Smith and Holladay, 2001). If fixation is used, perfusion of the vascular system or hollow parts of the specimen with 10% formalin, followed by immersion is an efficient method for fixation. Some routine fixation fluids may be used. However, those containing alcohols, glycerin, glycols and/or phenol should not be used. Such chemicals may yield a brittle specimen and/or may interfere with the curing process, as well as, precipitate on the specimen surface years later.

When using formalin for fixation, concentrations are calculated as follows: commercially available solutions of formaldehyde gas in water are typically 33%-37%. When preparing an X% solution for preservation, consider the 33-37% as a 100% stock solution, which will be diluted with water to the desired percent. Therefore, a 10% formalin solution is created by mixing 10pbv of the stock solution (33-37%) with 90pbv water. In the ideal case, specimens remain in the fixative just long enough for fixation. A shortened fixation time using a low percent of formalin may yield a specimen with more flexibility. However, even with longer fixation, the specimens may be plastinated and will still be useful. Hollow organs retain their shape better with harder fixation. Brains always need to be fixed hard.

Redissection: During the process of plastination, due to inevitable artifacts, specimen quality likely will diminish. Therefore, only the best dissected specimens will yield superior results (Poorly dissected specimens introduced into the process = garbage output!). After fixation is complete, check the specimen and trim any loosened and/or unwanted fragments. Special attention should be given to the form/shape of the specimen and to surface detail of the specimen.

Cut edges (limbs for instance) should remain 1-2cm longer than the desired final specimen. After curing, the correct length can be determined and the final cut made which will yield a neater surface. Small holes (2mm) can be drilled in long bones every few centimeters in obscure positions to allow better defatting of the medullary cavity of the bone. For better defatting, the

marrow cavity can be flushed (water or ethanol) by introducing a small nozzle into one of the drilled holes.

Dehydration

Principles of dehydration: Dehydration replaces tissue fluid/water (both inter- and intra-cellular) with an organic solvent. This solvent must be miscible with water and preferably volatile enough to serve as a volatile intermediary solvent which is the key to the impregnation step Common dehydrating solvents include acetone and alcohols. Common volatile intermediary solvents include acetone and methylene chloride (dichloromethane) (MeCl).

Although alcohols are good dehydrating solvents, they are not suitable for use as the volatile intermediary solvent because their vapor pressure at -15°C is too low to be extracted gradually and continually. Although methylene chloride (MeCl) is a superb volatile intermediary solvent, it is not miscible with water and therefore not a dehydrating solvent. For these reasons and others, acetone has become the universal dehydrating solvent for the plastination process although alcohols may be used. If alcohol is chosen for the dehydrant, after dehydration the specimen must then be saturated with acetone or MeCl to serve as the intermediary solvent.

During dehydration, some shrinkage always occurs. Shrinkage is usually less in long-term formalin-fixed specimens or specimens containing less fat. Shrinkage is greater in fresh tissue specimens and those containing an increased percentage of fat. Dehydration in -25°C acetone decreases shrinkage because the water in the specimen freezes and stabilizes the form, structure and size of the specimen. Low temperature, -25°C, does not decrease the quality or rate of dehydration. This technique is known as "freeze substitution" and is the preferred method of dehydration for plastination (Schwab and von Hagens, 1981; Tiedemann and Ivic, 1988; Brown et al., 2002).

Preparation for dehydration: Before starting dehydration, be sure that glycerin, glycol or phenol, often components of embalming fluids, are not in the specimen. To remove these chemicals, immerse the specimen in 50% ethanol for seven days and stir the solution and specimens twice a day. After one week, rinse the specimen in running tap water for a few hours and then place it in a fresh 50% ethanol solution for seven more days and stir twice daily. After the second ethanol bath, rinse the specimen in tap water for one week to remove the ethanol. Ethanol removal is necessary as its specific gravity is the same as that of acetone. Specific gravity is used to determine acetone purity. Therefore, ethanol in the specimen will give a false increased purity reading of the acetone dehydration bath.

Specimens fixed in formalin without additives, need to be rinsed in tap water for two to four days to remove the formalin. If not carried out, formalin is leached out of the specimen into the acetone during dehydration. During acetone distillation, paraformaldehyde precipitate will form in the distillation coil and may eventually clog the coil.

Dehydration equipment and chemicals for dehydration:

- Deep freezer: Explosion-proof/safe or household freezer modified by removal of compressor and motor and placing them outside the room. Also the light bulb is removed.
- Chemical resistant receptacles with a lid: Large enough to hold the specimens and one aliquot of acetone (10 times the volume of the specimen).
- Perforated specimen basket with lid: Fit inside the above receptacle to contain and submerge the specimens in the dehydrant. Both the receptacle and basket must be constructed of acetone/ methylene chloride resistant material (stainless steel or high density polyethylene) (Fig. 2).
- Acetonometer or alcoholmeter: [To measure acetone purity of the dehydration bath (either analog or digital)]. Acetonometers are temperature dependent, often +20°C or -10°C (Fig. 3). Therefore, as acetone purity is determined, the temperature of acetone must be monitored and correspond (or be warmed or cooled) to the calibrated temperature of the acetonometer.
- For large and/or dilation projects: Peristaltic pump or gravity reservoir, silicone tubing and connectors to perfuse hollow specimens.
- Acetone: Three aliquots of 10 times specimen volume.

Dehydration procedure: Precool the specimens to 5°C in tap water. After pre-cooling: Drain excess water from the specimens. Arrange specimens in an anatomical position in the specimen basket. Fill hollow structures/organs with cold acetone. Assure proper anatomical position. Do not attempt to over dilate as cold acetone freezes the specimens and will not allow further dilation.

Close the basket lid and submerge specimens into an aliquot of -25°C acetone (90-100%) (Fig. 4) and place in the freezer. An acetone to specimen ratio of 10:1 is desirable and fool proof methodology.

After six days, check and record the purity of the acetone. Fill an appropriate sized cylinder with the used acetone, monitor temperature (warm or cool acetone as necessary), insert and read the acetonometer (Figs. 5, 6). Water tends to settle to the bottom of the receptacle.

Therefore for an accurate purity reading, gently stir or agitate the used acetone prior to the purity reading. On day seven (the usual time needed for the acetone to equilibrate in the specimen and vat), check and record acetone purity. If this purity is the same as or similar to the purity on day six, raise the specimens and allow excess surface acetone to drip from the specimens. Caution, do not let the specimen surface become dry. Place specimens in a new aliquot of -25°C acetone and back into the freezer. Filter the used acetone and use for your next specimens or recycle/distill. If purity has decreased, monitor acetone purity daily until it remains the same and then change to new acetone.

Repeat procedure weekly until the acetone concentration in the dehydration bath remains >99%, which will also be the concentration in the specimen when equilibrium is reached after 7 days. At this point, dehydration is considered to be complete.

Principal: If pure acetone is used and a 1:10 specimen:acetone ratio has been maintained, a dehydrated specimen can be assured after 3 weekly changes. For large specimens it may be desirable to check purity again at 8 or 9 days to assure equilibrium has been reached.

Tip: Stirring or agitating the acetone/specimens daily may hasten dehydration with equilibrium resulting in 4 or 5 days.

Defatting (degreasing)

Defatting is the removal of excess fat/lipid from the specimen. Lipid does not impregnate well. Too much lipid may decrease specimen durability. Fat is translucent. However, nervous tissue should not be defatted since lipid (myelin sheath) is a major integral part of neural specimens. Excess defatting of neural specimens causes excessive, unacceptable shrinkage.

Procedure: When specimen acetone content/acetone purity is >95%, remove receptacle with the >95% dehydrated specimens from the deep freezer to room temperature (RT) for several days to weeks. For better safety, place receptacle with the acetone and specimens in or by a fume hood or room exhaust. The lid must fit tightly to avoid excess acetone loss by evaporation. Sealing the lid with plastic wrap (foil) will decrease evaporation loss of solvent. Stirring or agitation will likely hasten defatting, but is not necessary. Check weekly for color change of acetone from clear to yellow. Lipid turns the transparent acetone yellow. Transfer the specimens to a fresh, 98% - 100% acetone bath at room temperature to continue degreasing until the fat on the specimens has begun to loose its white color (becoming slightly opaque). Repeat the above procedure weekly, until the desired decrease in lipid content is reached (between white and opaque), 2-6^{+} weekly changes. When degreasing is judged complete, place the specimens in the silicone impregnation-bath. Place the fatty/yellow acetone in a -25°C freezer over night. The dissolved fat will congeal and much fat can be filtered from the acetone using a cloth/towel (Fig. 7). The defatted/filtered acetone is reused or distilled.

Methylene chloride [dichloromethane (MeCl)] is a powerful degreaser (Figs. 8, 9). Remove dehydrated specimens in their basket from the 99% acetone or alcohol. Drain excess dehydrant from specimens and place specimens in an appropriate aliquot of MeCl (enough to cover the specimens) for 2 - 7^{+} days, until the desired defatting is accomplished. MeCl quickly renders opaque fat to semitransparent. The defatted specimens are then placed into the impregnation-bath. MeCl is hazardous and must be used in a ventilated hood.

Day 1	Pre-cool specimens to 5°C over night.
Day 2	Place specimens in an aliquot of -25°C acetone (>90%). 1:10 (specimen:acetone) ratio.
Day 9	Measure & record purity of used acetone. Place the specimens into a fresh aliquot of new (99-100%) acetone (-25°C).
Day 16	Measure & record purity of used acetone. Place the specimens into a fresh aliquot of acetone (-25°C). If purity is >95%, sit receptacle with specimens and acetone out to room temperature (RT) for degreasing.
Day 23	Check acetone purity and color, as well as, fat color: a. If purity is >98% and fat is negligible, place specimens into the cold impregnation-mix. b. If purity is <98% and fat is not opaque and the acetone is yellow, place specimens into a new aliquot of fresh acetone or MeCl.
Day 30	Check acetone purity and color, as well as, fat color: a. If purity is >98% and fat is negligible or opaque, place specimens into the cold impregnation-mix. b. If purity is <98% and fat is not opaque, place specimens into a new aliquot of fresh RT acetone or MeCl.
Day 30 + X	When acetone purity is >98% and specimen is adequately defatted, place specimens into the cold impregnation-mix.

Table 1. Dehydration and defatting schedule.

Forced impregnation

Principles of forced impregnation: Forced impregnation is the replacement of the volatile solvent in a biological specimen with a curable polymer. For this to occur, the

volatile intermediary solvent must be miscible with the silicone polymer and must have a sufficiently high vapor pressure at -15°C to be gradually, continuously and completely extracted during the impregnation step of the plastination process. As the principal of dehydration is to replace tissue water/fluid with acetone, the impregnation principal is to replace the volatile intermediary solvent (acetone or MeCl) with the reaction-mixture of S10/S3. However, the reaction-mixture is too viscous to come to equilibrium with the solvent (acetone/MeCl). Therefore, a force (vacuum) is needed to get the reaction-mixture inside the specimen (hence the term forced impregnation).

The solvent filled (acetone/MeCl) specimens, in the specimen basket (Fig. 10), are submerged in the liquid silicone reaction-mixture in the vacuum kettle (plastination chamber) which is in the -15°C deep freezer. Applying vacuum to the reaction-mixture and the specimens, causes the solvent to vaporize/boil (at a known pressure) and leave the specimen, pass through the reaction-mixture and finally the vaporized solvent is pumped out through the vacuum pump exhaust. Vaporization of the acetone from the specimen leaves a tissue void or negative pressure inside the specimen and the reaction-mixture is drawn into the specimen. Hence, for this exchange to take place, there must be enough time for the viscous polymer-mixture to enter the cells of the specimen. Therefore, if the pump speed is too great and generates excess vacuum too quickly, too much acetone will leave and the tissue will collapse. The collapsed tissue will not allow the viscous reaction-mixture to enter. This results in shrinkage and desiccation of the specimen. Hence, two fine adjustment needle-valves (Biodur™ HI 14) are recommended for delicate control of pressure.

Impregnation equipment:

- Deep freezer, explosion-proof/safe or household freezer made safe by removal of and placement of compressor and motor in adjacent room.
- Vacuum chamber with a transparent lid [tempered glass or polycarbonate (thick enough to withstand one atmosphere decrease in pressure)] (Fig. 11).
- Vacuum pump - slow pump speed is best, with the capacity of reaching an end pressure near 0mm Hg (Fig. 11). Oil type pump is recommended. Dry pumps may not reach appropriate final vacuum.
- Vacuum gauge, tubing and fine adjustment needle-valves (Figs. 11, 12).
- Bennert mercury (Fig. 12) or digital manometer.
- Specimen basket (Figs. 2, 11).

Reaction-mixture preparation: Biodur™ S10 or S15 polymer is mixed with S3 (Catalyst with Chain extender) at 100:1 to prepare the reaction-mixture. Thorough stirring is necessary, as per the manufacturer's instructions for 10 minutes at a medium speed. Using an electric drill and stirrer (paint) is recommended. However an old fashioned stir stick will work. During mixing, the reaction-mixture will first turn opaque, then clear. This polymer-catalyst-mix should be deaerated. Place the mix in a vacuum chamber and reduce pressure to 30cm Hg. The air will boil out and then the reaction-mixture is ready to be used at -15°C for impregnation or to be stored in the deep freezer (preferably less than -25°C).

Vacuum adjustment: Speed of lowering the pressure in the vacuum chamber is dependent on temperature and/or viscosity (age) of the reaction-mixture. At higher temperature or lower viscosity, pressure can be lowered faster because of the decreased viscosity of the polymer-mix. The following scheme, is a rule of thumb for cold temperature (-15°C) impregnation. If in doubt, go slower, to prevent shrinkage.

Impregnation regimen:

Day 1: The dehydrated and degreased specimens are taken from the solvent (acetone or MeCl), excess solvent is drained and the dehydrated solvent filled specimens are placed in the cold polymer reaction-mixture. Submerge immediately to prevent solvent evaporation from the specimens and drying of their surface. Be sure all specimens are submerged, acetone-soaked specimens tend to float in the reaction-mixture. Hence, a grid to keep specimens submerged or a specimen basket to contain and submerge the specimens is necessary. Fill hollow organs with the cold reaction-mixture (heart chambers, gut etc.) or cut small ports in organs to prevent collapse and alteration of their shape during the lowering of pressure and to keep polymer in contact with all surfaces of the specimen (Fig. 14). Place the lid (glass) on the vacuum chamber and let the specimens accommodate/equilibrate over night.

Day 2: Turn on the vacuum pump and run for 10 minutes to warm to operational temperature. A hot pump will prevent condensation of acetone or MeCl vapors in the pump oil. Seal the chamber by closing the vacuum adjusting unit (needle valves) and applying the vacuum. Once the seal is accomplished, allow the pressure to lower slowly to 22cm/9in Hg. The Bennert mercury manometer will commence to measure pressure near this pressure level (Fig. 12). Stabilize pressure at this level by opening the needle valves incrementally and slowly, as needed, to stabilize the pressure near 22cm/9inches Hg). During this time small bubbles (trapped air) will rise through the polymer-mix. The goal from this point will be to: Daily, decrease pressure 1/3 of the current daily

pressure level until the solvent begins to vaporize.

Day 3: Slowly decrease pressure 1/3 of the current daily value (22cm/9in) to 14cm/5.5in Hg.

Day 4: Continue to slowly decrease pressure 1/3 the current daily value to 9cm/3.5in Hg.

Day 5: Continue to slowly decrease pressure 1/3 the current daily value to 6cm/2in Hg (as suggested in the table below). Daily decrease of pressure is continued until 1cm bubbles continually rise to the polymer surface and burst.

The goal is to reduce pressure slowly (1/3 of the current reading) over a four to six day period to the point where the intermediary solvent will begin to vaporize/boil and leave the tissue at a slow steady rate. This will allow the reaction-mixture to enter the tissue void created by the vaporizing and exiting acetone. The pressure at which the solvent's boiling point is reached will vary with temperature and the solvent used. When at this point, 1 cm bubbles will arise continually to the surface and burst. Before this point a few sporadic bubbles will rise but likely sit on the polymer surface. For acetone, this steady extraction of solvent @ -15°C occurs around 3cm Hg pressure or 1.5cm Hg pressure @ -25°C. For MeCl @ -15°C, extraction occurs at a higher pressure 7cm Hg pressure or around 3.5cm Hg pressure @ -25°C (Pereira-Sampaio et al., 2006).

Day 6: Pressure is now close to the boiling point of acetone. If bubbles are rising to the polymer surface and bursting, do not decrease pressure. However, if bubbles are not rising, slowly decrease pressure 1/3 of the current value daily to 4cm/1.5in) as suggested in table 2 below and 1cm bubbles will likely rise to the polymer surface and burst.

Rule: If bubbles are actively rising to the top of the polymer and bursting, do not decrease pressure! It is better to decrease pressure too slow than too fast. Once bubbles are active (solvent vaporizing), decrease of pressure is not recommended until bubble activity slows. Bubbles should rise slowly but continually as simmering water, not as rapid boiling water. Acetone removal and hence polymer impregnation at cold temperature will take 3 to 5 weeks depending on volume of specimens and pump speed. **If bubbles cease to rise, slowly and incrementally close the needle valve to decrease pressure until active bubbles start to rise again. Usually it is necessary to lower pressure only 1-2mm Hg to continue active bubble production. It may take a few minutes after incremental valve closure before bubble production is observed.

Evacuation of acetone/solvent too quickly will result in incomplete impregnation of the specimen with the polymer-mix and shrinkage.

Impregnation is complete when the needle valves are closed and no more acetone bubbles appear at the surface of the reaction-mixture for several hours and/or near zero pressure has been maintained for a few days.

Day 1	Load specimens and allow to equilibrate over night.
Day 2	Start pump, slowly decrease pressure to atmosphere to: 22cm/9in Hg.
Day 3	Slowly decrease pressure, 1/3 current pressure reading to: 14cm/5.5in Hg.
Day 4	Slowly decrease pressure, 1/3 current pressure to: 9cm/3.5in Hg. Air bubbles form but not continually rising.
Day 5	If no bubbles rising, slowly decrease pressure 1/3 current pressure to: 6cm/2in Hg.
Day 6	If bubbles actively rising to the surface and bursting, do not decrease pressure. If no bubbles, slowly decrease pressure 1/3 current pressure to: 4cm/1.5in Hg.
Day 7 - Day X	Active bubbles, Do not decrease pressure! When bubbles cease or slow dramatically, decrease pressure 1cm Hg.

Table 2. Impregnation schedule.

Specimen removal: Turn off vacuum pump. This is a good time to drain the oil from the pump while warm and refill with new oil. It may be beneficial (but not necessary) after this oil change to run the pump for one hour with the fresh oil and then drain and change the oil once more. This aids removal of any residue of acetone and/or MeCl from the pump. Vacuum oil is inexpensive. Vacuum pumps are expensive.

Open valves and allow vacuum chamber and specimens to return to atmospheric pressure slowly. A sudden release of pressure will allow the Hg in the Bennert manometer to rush to the top of the glass tube causing the glass to break. It may be beneficial to leave the specimens in the reaction-mixture for 24 hours to equilibrate.

Day 1: Raise specimen basket from the reaction-mixture and let the excess polymer-mix drain from the specimens into the vacuum chamber. Turning the specimens a few times that day will allow more complete drainage of polymer-mix from the surface, crevices and hollow parts of the specimens. Initially, this may occur in the deep freezer. Specimens may be stored or drain indefinitely in the freezer if schedule does not permit manicuring of the specimens.

Day 2: Remove specimens from the freezer and allow draining to continue into a container at RT. This captured polymer-mix should be returned to the cold impregnation chamber daily.

Day 3^{+}: Place specimens on absorbent paper. Turn, reposition/shape and dilate hollow specimens with air or absorbent toweling daily. They can continue to drain for several days to weeks at room temperature. Change absorbent paper as needed and wipe the surface of specimens and manicure daily.

Day 4 or X: Once the specimen is no longer oozing silicone, it is ready for gas curing (cross-linking).

Curing/Hardening/Cross-linking

During curing, the impregnation reaction-mix within the specimen is cross-linked and the specimen made dry. This is a two-step process consisting of chain extension and cross linkage of polymer.

Chain extension: Chain extension of the silicone molecules is an end to end alignment, thus forming longer chains via the chain extender portion of the S3 impregnation-mixture which is now in the specimens. This first reaction, known as "pre-curing", results from the reaction of the S3 with the S10. This is also known as "slow cure" (Table 3). It is advantageous to pre-cure for at least two or three days. However, longer pre-curing is even better and may yield more flexible specimens. Specimens may remain at RT for a few days to a few weeks to allow maximum chain extension. During this time the specimens must be positioned anatomically correct and dilated to assure correct position as chain extension progresses. Theoretically, chain extension starts when the S3 (catalyst & chain extender) and S10 polymer are mixed. However, this reaction is slowed dramatically by cold temperature (less than -15°C). The polymer reaction-mixture may be kept for a few years in the cold (less than -25°C). Longer chains result in more viscous polymer. At room temperature, the elongation occurs at an increased rate. In six to ten months, at RT, the reaction-mixture will become too viscous for impregnation. Therefore, it is advisable to have the specimens positioned anatomically correct when pre-curing at room temperature (Table 3).

Cross-linking: Cross-linking or connecting the silicone polymer molecules side to side, forming a firm 3-D meshwork of the silicone polymer, is caused by the S6 (cross-linker). The catalyst (S3) prepares the S10 molecules to react with the S6 cross-linker. The S6 is more reactive in its vaporized (gaseous) state, hence the term "gas curing" is used. The vaporized S6 diffuses onto the impregnated specimen's surface. The cross-linking reaction starts on the specimen's surface and proceeds inward to the depths of the specimen (von Hagens, 1986; Weiglein & Henry, 1993). Larger specimens may take longer to cure. Cross-linking may be started soon after removal of the specimens from the deep freezer and polymer-mix with minimal draining time = fast cure (Table 4). However, it is desirable to let specimens drain a few days before gas curing. Fast cure at this timing will likely take at least one week to complete. Or cross-linking may be done after several weeks of draining and manicuring at room temperature. This is called pre-cure or "slow cure" (Table 3). After a long slow cure, cross-linking likely may take only one or two days.

Equipment for curing:

- Curing chamber: Air-tight closed container, large enough to contain the specimens (Fig. 15).
- Aquarium pump or small ventilator (fan) spark free: To volatilize the S6.
- Desiccant ($CaSO_4$): To remove moisture from the air around the specimen in the chamber.
- Compressed air connection, tubing and connectors: To dilate specimens and/or to evacuate polymer trapped in the organ.
- Absorbent paper: To blot excess polymer.

Cross-linking regimen for fast cure: At the beginning of the week, it is beneficial to start early in the morning to manicure specimens preparing them for cross-linking (expose to S6). This will assure at the end of the week the surface of the specimen is cured with no silicone oozing and a dry surface.

Dilate all specimen cavities that are collapsed, using compressed air or toweling to restore normal anatomical size and shape. Slowly inject 0.1 to 0.3ml S6 into the compressed air hose/tubing to start curing of the interior of the specimen. This will help establish its anatomical shape during the curing process. Repeat S6 injection via the tubing daily as often as necessary.

Place the desiccant ($CaSO_4$) and specimens into the curing chamber on absorbent paper. Assure normal anatomical shape and position of the specimen. Place a small container of S6 (10-20cc) in the curing chamber. Close chamber and vaporize S6 via an aquarium pump or fan for a few minutes 2 times a day.

Curing starts on the specimen's surface as the S6 vapor contacts the specimen. The first day it is necessary wipe the surface every 2-3 hours and turn the specimen to prevent silicone drips or runs. After that, manicure the surface and turn the specimens twice a day. The specimens may be placed in the deep freezer overnight to slow the curing process if seeping of polymer has been excessive. The next morning (or when it is convenient) the specimens are placed back in the curing chamber.

After a few days, depending on surface concentration of S6 on the specimen surface and the degree of chain extension, the surface of the specimen will become tacky and then dry. Once the surface is sealed (dry), the specimen may be used. However, the interior of the

specimen may not be cured. The specimen must be stored in an airtight plastic bag or in the curing chamber to assure complete internal hardening of the polymer and hence eliminate oozing of polymer later on. Avoid direct contact of the liquid S6 with the specimen. Depending on the size of the specimen this final deep curing may take a week or two. After thorough curing, the specimen is ready for permanent use (Table 4).

Day 1	Bring specimens to atmospheric pressure and allow excess polymer to drain in the cold.
Day 2 or longer	Bring specimens to room temperature to drain excess polymer.
Day 3	Manicure specimen surface and dilate and/or position anatomically. Allow specimens to drain.
Day 4- X (Several days or weeks)	Manicure specimen surface and dilate and/or position anatomically. Allow specimens to drain. At the appropriate time: Expose specimens to gas cure agent (S6) and manicure surface.
Day X plus 1	Manicure surface and Expose specimens to gas cure. Follow fast cure regime from day 4.

Table 3. Slow/pre-curing schedule prior to fast-curing.

Day 1	Bring specimens to atmospheric pressure and allow cold draining.
Day 2	Bring specimens to room temperature to drain excess surface polymer.
Day 3	Manicure specimen surface and dilate or position anatomically. Allow excess polymer to drain from specimens.
Day 4 or longer	Manicure surface and Expose specimens to gas cure agent (S6).
Day 5	Manicure surface. Expose specimens to gas cure agent (S6).
Day 6	Likely surface will be nearly dry. Manicure the surface and expose to S6.
Day 7	Likely surface will be dry. Leave in gas chamber or enclose in bag to assure curing to the depths of the specimen.
Day 8	Use specimen as desired or leave contained in the closed environment.

Table 4. Fast-curing schedule.

Results

Biodur™ S10 plastinated specimens serve as the gold standard among silicone plastinated specimens as well as specimens preserved by other mechanisms. Surface clarity is superb. Thinner specimens exhibit flexibility. All specimens are durable, free of offensive odor and dry. They may be used as teaching aids both in the class room and the clinical setting. They may be used as a library of specimens for normal, exotic and pathological anatomy. They are irreplaceable public relation tools.

Discussion

Specimens produced by the Biodur™ S10 silicone methodology are of superb quality (Figs. 1, 8, 9, 14, 16-21). Surface clarity is perfect and some flexibility is associated with thinner specimens. All specimens are durable, free of offensive odor and dry. They may be used as teaching aids both in the class room and the clinical setting (Bickley et al., 1981; Latorre et al., 2001). They have been used as a library of specimens for normal (Figs. 1, 8, 9, 14, 18-20), exotic (Figs. 17-19) and pathological anatomy (Fig. 16, 17) (Bickley et al., 1981; Latorre et al., 2001, Sakamoto et al., 2006).

Note concerning impregnation and polymer level: Polymer level over 50cm/20in deep poses a potential problem for extracting the intermediary solvent from the depths of the polymer-mix. Intermediary solvent is more difficult to extract from the specimen due to the pressure exerted by the depth of polymer overlying the specimen (Henry and Thompson, 1992; Henry, 2005a; 2005b; von Horst, 2006). Therefore, depth of polymer should not be excessive. A stronger pump will be necessary for deep polymer reservoirs.

To efficiently utilize available acetone, less that pure acetone (>80%) may be used for the first and second acetone baths. However, this will necessitate more changes of acetone. Principal: Always place specimens into acetone of a higher percent than that from which they were taken.

The Biodur™ S10 or S15 polymer and Biodur™ S3 catalyst-mix may also be used for impregnation at ambient temperature. The protocol is similar to the above description for cold impregnation. The exception is that impregnation is carried out at room temperature. Specimen preparation and dehydration are routine. Impregnation is modified and carried out at room temperature. The standard impregnation-mix is prepared at the 100:1 [S10 (polymer):S3 (catalyst/chain extender)] ratio. However, the impregnation-mix must be stored at -25°C when not in use. Impregnation time is shortened by two-thirds and often may be completed in one week since a more rapid boil (solvent extraction) is carried out. This is possible, since the polymer is at room temperature and is less viscous. Thus the polymer can enter the cells at a faster rate. Solvent vaporization and hence impregnation commences around 22cm/10in Hg pressure, the vapor pressure of acetone at room temperature (25°C). MeCl's vapor pressure at RT is higher (43cm/17in Hg) and hence impregnation of specimens saturated with MeCl will commence at a

Figure 1. Hydrostatically, dilated ascending colon and cecum of equine foal. S10 Cold-temperature.

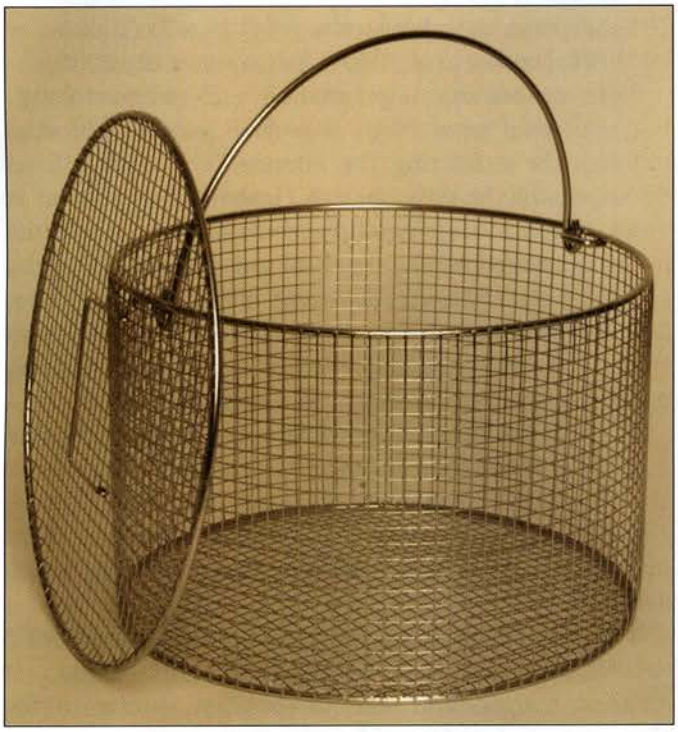

Figure 2. Specimen basket with lid.

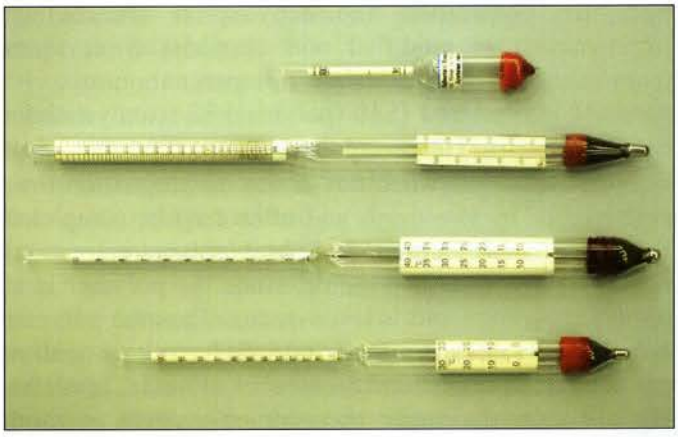

Figure 3. Assortment of acetonometers.

Figure 4. Dehydrating specimens in basket being transferred to fresh acetone.

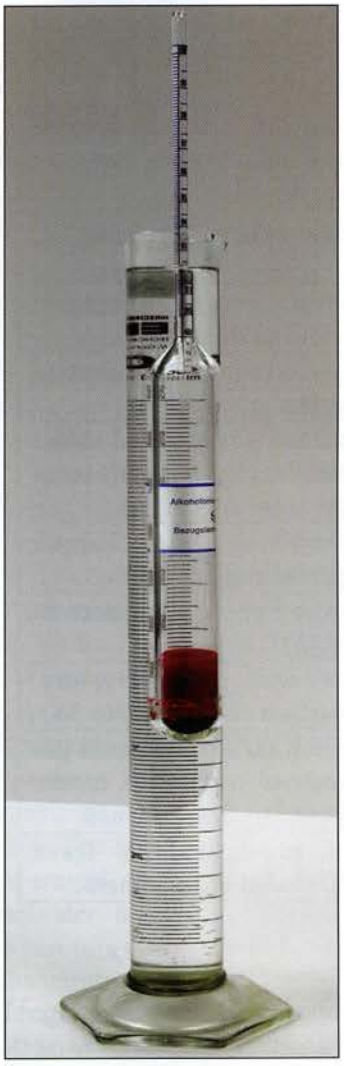

Figure 5. Monitoring acetone purity with acetonometer calibrated for +20°C.

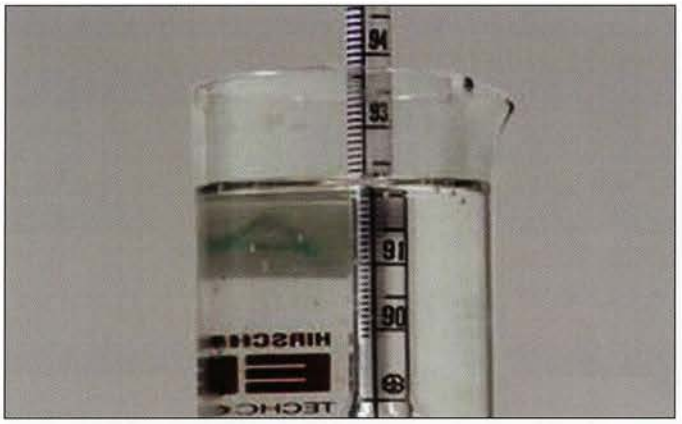

Figure 6. Acetonometer registering 92%.

Figure 7. Degreasing acetone: Left - prefiltration; Right - Congealed fat after filtration.

Figure 8. Degreasing: Horizontal section of caprine illustrating less than desirable acetone degreasing. S10 Cold-temperature.

Figure 9. Transverse section of domestic feline at the level of the sacroiliac joint plastinated with the S10 cold temperature method illustrating optimum degreasing.

Figure 10. Dehydrated/degreased specimens in silicone polymer awaiting the grid to keep them submerged. S10 Cold-temperature.

Figure 11. Impregnation setup. Vacuum kettle with basket, gauge and needle valves; vacuum pump; Bennert manometer.

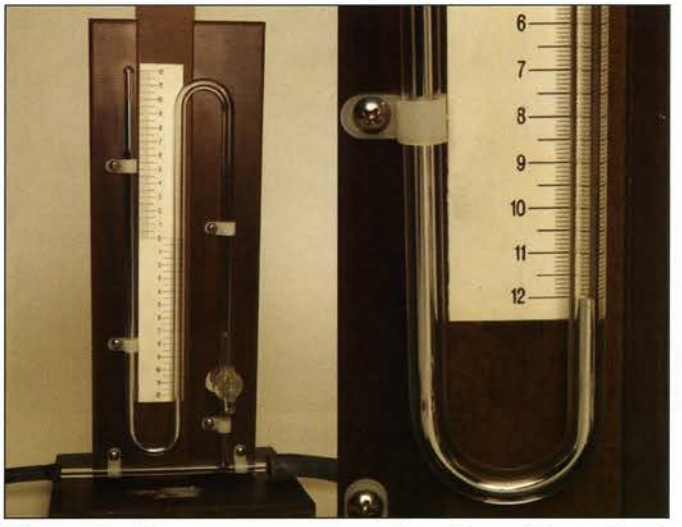

Figure 12. Bennert manometer reading 22cm (10cm+12cm) Hg pressure.

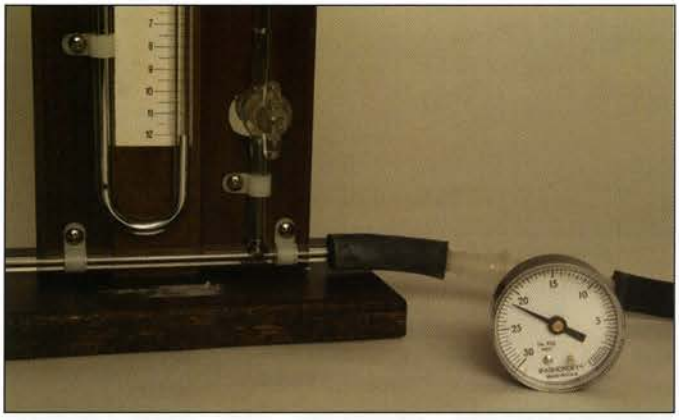

Figure 13. Bennert manometer reading 22cm Hg pressure and vacuum gauge showing 21.5 in gauge vacuum which is equal to 21.5cm Hg pressure.

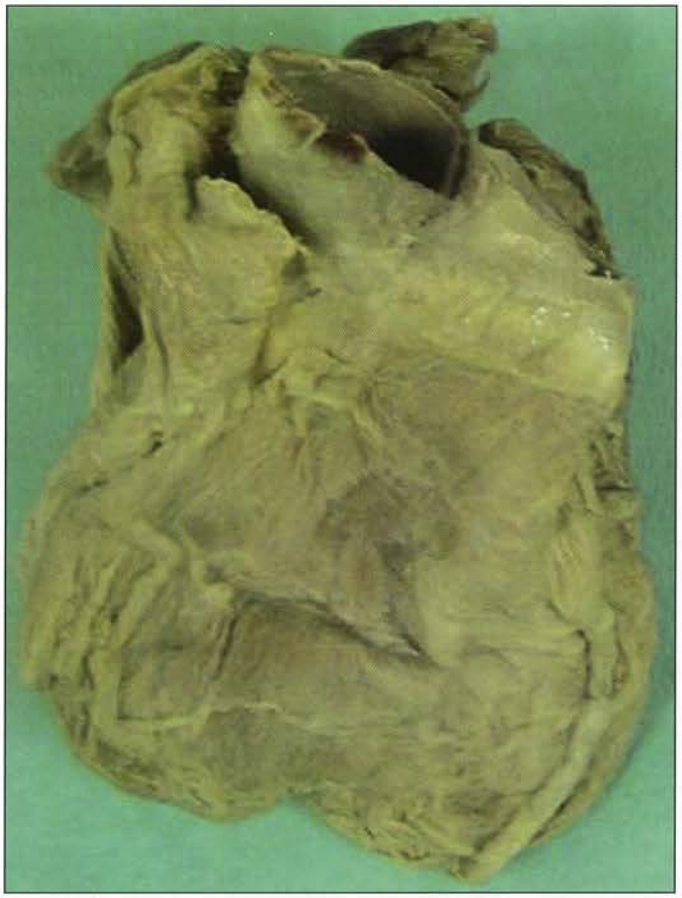

Figure 14. Collapsed, dilated human heart. S10 Cold-temperature.

Figure 15. Curing chamber with spark-proof ventilator.

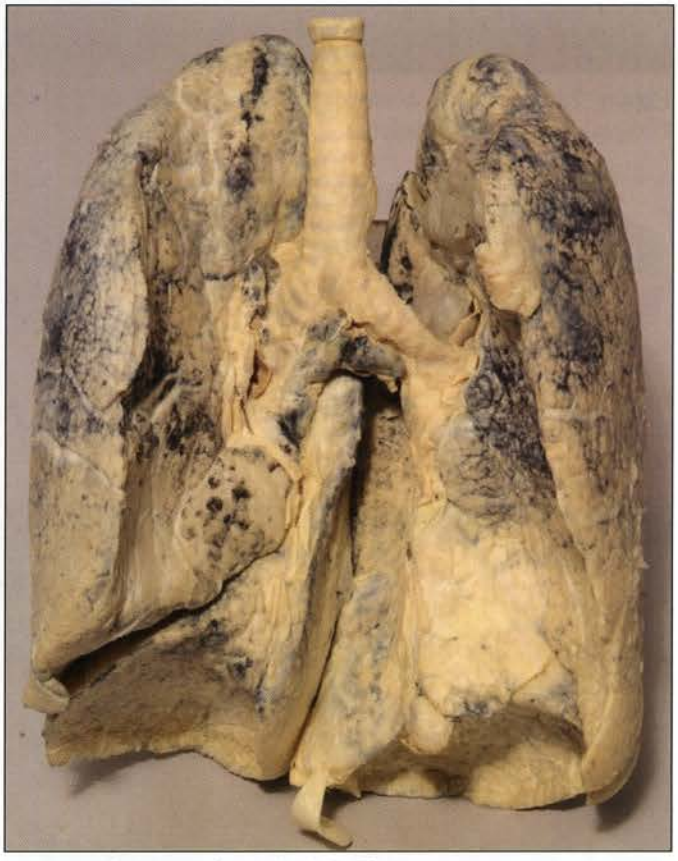

Figure 16. Human lung. S10 Cold-temperature.

Figure 17. Pathological specimens. Eagle foot/pes with avian pox. S10 Cold-temperature.

Figure 18. Boa constrictor. S10 Cold-temperature.

Figure 19. Close up of Figure 18.

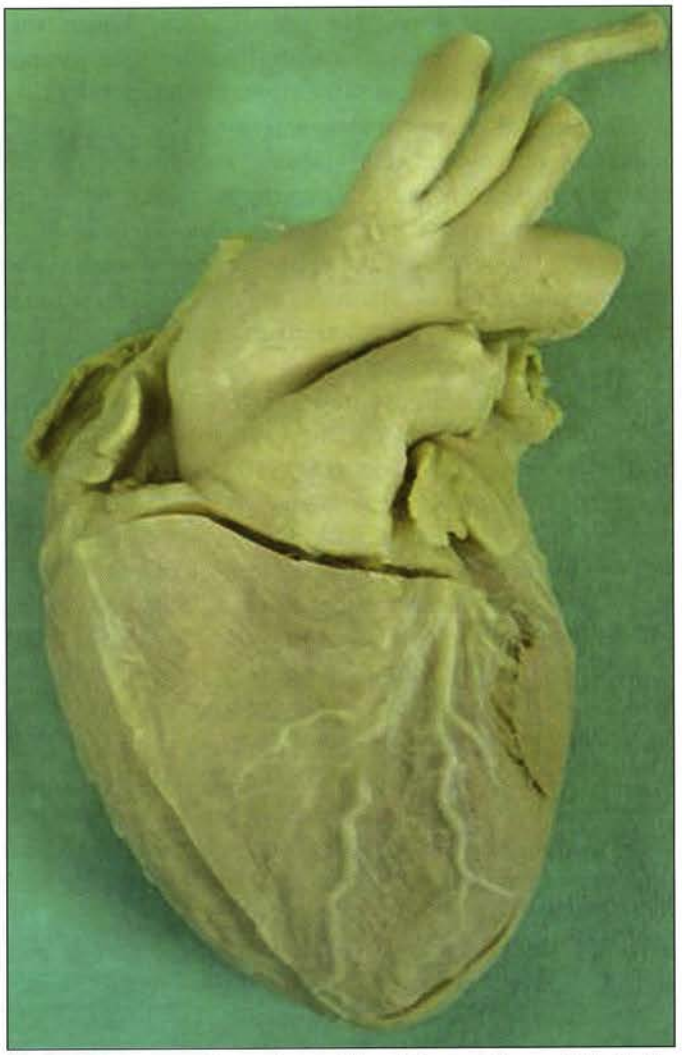

Figure 20. Noncollapsed, nondilated human heart. S10 Cold-tempertaure.

Figure 21. Jelly fish. S10 Ambient-temperature technique.

higher pressure, 43cm Hg pressure and hence this solvent will be removed easier than acetone. Vapor pressure of the solvent is proportional to temperature.

Draining of excess impregnation-mix is more rapid since the polymer is less viscous. Therefore, gas cure may commence the same day as impregnation ends. Gas cure may be via either slow or fast methodology. However, longer pre-cure is recommended. The polymer-mixture is stored at -25°C or colder immediately upon completion of impregnation and draining of the specimens.

Ambient-temperature impregnated Biodur™ S10 silicone plastinated specimens have the same ultimate quality as that of cold Biodur™ S10 silicone plastinated specimens. Surface clarity is perfect and some flexibility is associated with thinner specimens (Fig. 16). All specimens are durable, free of offensive odor and dry. They make excellent teaching aids both in the class room and the clinical setting (Bickley et al., 1981; Latorre et al., 2001). As they are accumulated, they can become a fantastic library of specimens for normal, exotic and pathological anatomy.

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Product distribution:

Biodur™ Products, Drs. Gunther von Hagens and Angelina Whalley, Rathausstrasse 11, 69126 Heidelberg, Germany. www.biodur.de, Ph: (49) 6221 3311-11, Fax: (49) 6221 3311-12.

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Silicone Plastination of Biological Tissue: Cold-temperature Technique North Carolina Technique and Products

R.W. HENRY

Department of Comparative Medicine, College of Veterinary Medicine, University of Tennessee, Knoxville, TN, USA.

**Correspondence to: Telephone:* (865) 974 - 5822; *Fax:* (865) 974 - 5460; *E-mail:* rhenry@utk.edu

Abstract: With the emergence of the Biodur™ S10 plastination process as the gold standard for preservation of biological tissue, similar products and processes have been developed. The North Carolina cold-plastination process is a modification of such. The alteration of the technique is when the chain extender is used: From during impregnation to prior to curing. The NCSX and XI polymers and additives (catalyst, chain extender, cross-linker) yield exceptionally beautiful and aesthetically pleasing plastinated specimens of similar quality to Biodur™ specimens. The North Carolina Cold-temperature technique is used for whole cadavers, organs or portions thereof.

Key words: **plastination; silicone; polymer; NCSX; NCSXI; NCSIII; NCSV; NCSVI**

Introduction

Silicone plastination replaces tissue fluid with a curable polymer. Several other silicone polymers and additives, other than the Biodur™ products, have been developed to carry out the silicone plastination process (Henry et al., 2001). The basic ingredients of each process are well known products of the silicone industry (Henry et al., 2002). Principally, methods of placing the silicone into the biological specimen are similar: Using an intermediary solvent (acetone) along with a decrease in pressure to remove the acetone (von Hagens, 1986; Henry and Nel, 1993; Henry, 2004). Removal of the solvent produces a tissue void and the polymer-mix is drawn into the cells. Each of these variants of the cold temperature silicone plastination technique produces high quality durable plastinates.

Chemicals used in this "generic" silicone-plastination process are similar to the ones used in the Biodur™ S10 plastination process:

- Acetone and possibly methylene chloride
- Silicone polymer
- Catalyst to prepare the silicone molecules for elongation and cross-linkage
- Chain extender to cause the silicone molecules to form longer-chain silicone molecules
- Cross-linker to form a 3-D meshwork of the elongated silicone molecules by side to side linkage

The general steps of silicone plastination are described earlier in this volume. This manuscript will highlight the differences with respect to this generic process which occur with impregnation and curing of the plastinated specimens.

Materials and methods

The basic steps of plastination are utilized for each plastination technique:

Specimen preparation, dehydration and defatting (degreasing)

Specimen preparation, dehydration and defatting are similar for all plastination methods. However, it is

necessary to prepare high quality dissected specimens to produce excellent plastinates. Please refer to: "The S10/15 Plastination Technique" for the basic, as well as, additional and more in depth information for specimen preparation and cold dehydration.

North Carolina products for silicone plastination:
NCSX (lowest viscosity): Silicone polymer
NCSXI (low viscosity): Silicone polymer
NCSIII: Catalyst
NCSV: Chain extender
NCSVI: Cross-linker

Forced Impregnation

Replacing the volatile solvent (acetone/methylene chloride) in a biological specimen or tissue with a curable polymer. For this to occur, the chemicals must meet similar criteria as the chemicals used in the "Biodur™ S10 Cold-temperature Technique".

Impregnation equipment: Similar to Biodur™ S10 requirements - Vacuum chamber (kettle), Vacuum pump (oil preferred), Vacuum gauge, Vacuum tubing, Needle valves, Manometer, Specimen basket and Deep freezer.

Preparing the reaction-mixture: NCSX or NCSXI polymer is thoroughly mixed with NCSIII (Catalyst) at 100:20-30 to prepare the impregnation reaction-mixture. The reaction-mixture is stored lower than -25°C and used for impregnation in a -15°C deep freezer. The impregnation-mix is not stable at room temperature and will become too viscous over a 6-10 month interval if stored and/or used at room temperature continually.

Adjusting the vacuum: Pressure is generally lowered by closing the air intake valves which are located in line between the vacuum kettle and pump. Speed of lowering the pressure in the vacuum chamber is slow and comparable to the Biodur™ S10 process. The NCSX and XI polymers have a lower viscosity than Biodur™ S10 and hence, impregnation may be faster. The impregnation-mixture is reactive and therefore needs to be kept at cold temperature. When monitoring bubble formation, a slow boil is recommended. As with any plastination procedure, if in doubt, reduce pressure slower, to prevent incomplete impregnation which will result in shrinkage.

Impregnation regimen: Similar to the Biodur™S10 Cold-temperature technique (Table 1).

Rule: If bubbles are actively rising to the top of the polymer and bursting, do not decrease pressure! It is better to decrease pressure too slow rather than too fast.

Once bubbles are active, solvent vapor pressure has been reached. More pressure decrease is not recommended until bubbling activity slows or nearly ceases. Bubbles should rise slowly but continually as simmering water, not as rapid boiling water. Acetone removal and hence polymer impregnation at cold temperature will likely take 3 to 5 weeks depending on volume of specimens and pump speed. **If bubbles cease to rise and the pressure is >3mm Hg, close the needle valve to decrease the pressure until active bubbles start to rise again. Usually it is necessary to lower pressure only 1mm to resume active bubble production (vaporization of solvent); but it may take a few minutes before bubble production is observed.

Evacuation of acetone/solvent too quickly will result in incomplete impregnation of the polymer-mix into the specimen and result in shrinkage.

Day 1	Load specimens. Allow to equilibrate over night.
Day 2	Start pump, Decrease pressure to: 18cm/7in Hg, by closing the needle valves incrementally.
Day 3	Decrease pressure to: 8cm/3in Hg, by closing the needle valve incrementally.
Day 4	Bubbles form but do not continually rise. Decrease pressure to: 5cm/2in Hg, by closing the needle valve incrementally.
Day 5	If bubbles actively rise to the surface and burst, do not decrease pressure. If no bubbles rising, decrease pressure to: 4cm/1.5in Hg.
Day 6	If bubbles actively rise to the surface and burst, do not decrease pressure. If no bubbles rising, decrease pressure to: 3cm/1in Hg.
Day 7 - Day X	Likely active bubbles, do not change pressure! When bubble formation slows dramatically or ceases, decrease pressure 1cm Hg.

Table 1. NCSX or NCSXI/NCSIII - Silicone/catalyst impregnation schedule.

Impregnation at -15°C is complete when needle valves are closed and no more acetone bubbles appear at the surface of the reaction-mixture for several hours and/or near zero pressure has been maintained for a day. Specimen removal: Similar to Biodur™ S10 Cold-technique. Drain excess surface polymer back into the impregnation kettle.

Curing/Hardening/Cross-linking

Equipment for curing:

- Similar to the Biodur™ S10 technique. However, the chain extender (NCSV) is utilized separately after impregnation, prior to cross-linking.

- Closed environment for NCSV application - The same type or same chamber as for cross-linking is used: Air-tight closed container, large enough to contain the specimens.
- Aquarium pump or small ventilator (fan, spark free): To volatilize the NCSV or NCSVI.
- Desiccant ($CaSO_4$): To remove moisture from the air around the specimen in the curing/chain extension chamber.
- Compressed air connection, tubing and connectors: To dilate specimens and/or to evacuate polymer trapped in the organ.
- Absorbent paper: To wipe excess polymer-mix from the specimen surface.

Curing, hardening and cross-linking the reaction-mix within the specimen to make the specimen dry is a two-step process.

Chain extension of the silicone molecules is end to end linkage of the silicone molecules. After the impregnated specimens have been drained of their excess polymer and wiped, the chain extender (NCSV) is applied via vaporization of the NCSV in a closed environment for two or three days. During this time, longer silicone molecules are forming. After exposure of NCSV to the specimens, the specimens should remain at room temperature for a few days to a few weeks to allow maximum chain extension (slow cure). During this time the specimens must be positioned anatomically correct and dilated to assure correct position as chain extension progresses.

Cross-linking or connecting the silicone polymer molecules side to side, thus forming a firm 3-D meshwork of the silicone polymer, is caused by the NCSVI component. This reaction and process is similar to that of Biodur™ S10 cold-technique. The drained and chain lengthened specimens are place in a closed environment with 10 - 20ml of NCSVI (cross-linker). The NCSVI is volatilized for 5 -10 minutes which commences the cross-linking of the silicone on the surface of and in the specimen.

Results

The NCSX or XI cold-impregnated specimens have the same great qualities of the Biodur™ S10 cold-technique specimens, including clarity of surface detail (Fig. 1). They are dry, durable, free of offensive odors and thin specimens exhibit some flexibility. North Carolina plastinated specimens are the real specimen and not a model (Figs. 1-3). NC cold-temperature specimens are used as teaching aids both in the class room and in clinics. When assembled in a group, they make an impressive library of specimens of normal, exotic and pathological anatomy. Silicone preserved specimens are useful in research, especially to preserve findings.

Day 1	Bring specimens to atmospheric pressure and allow to drain in the cold chamber.
Day 2	Bring specimens to room temperature to drain. Return drained impregnation-mix to freezer daily.
Day 3	Allow specimens to drain. Manicure specimen surface and dilate or position anatomically. Apply the chain extender NCSV via vaporization.
Day 4	Expose specimens to NCSV chain extender and manicure surface.
Day 5	Allow specimens to slow cure for days to several weeks.
Day 6 or longer	Expose specimens to gas cure NCSVI and manicure surface.
Day 7 or X + 1	Surface will be nearly dry. Manicure the surface and expose to NCSVI.
Day 8 or X + 2	Likely surface will be nearly dry. Manicure the surface and expose to NCSVI. Leave in gas chamber or enclose in plastic bag or container to assure curing to the depths of the specimen.
Day 9 or X + 3	Likely surface will be dry. Leave in gas chamber or enclose in plastic bag or container to assure curing into the depths of the specimen.
Day 10 or X + 4	Use specimen as desired or leave contained in the closed environment.

Table 2. Chain extension and curing schedule for NCSX or NCSXI silicone polymer.

Discussion

NCSX or NCSXI cold (Figs. 1-3) or ambient-temperature (Fig. 4) impregnated specimens have the great qualities of specimens produced by the Biodur™ S10 ambient and cold-techniques, as well as superb clarity of surface detail (Smodlaka et al., 2005). They are dry, durable and free of offensive odors. As well, flexibility is inversely proportional to specimen thickness. Both cold and ambient-temperature specimens have been used as teaching aids both in the class room and the clinical setting (Latorre et al., 2001). Also, specimens have been used to compile a library of specimens for normal, exotic and pathological anatomy (Henry, 2005a; Sakamoto et al., 2006) and in research.

It should be understood that in cold, impregnation, using a deep pool of polymer >40cm, makes it difficult

Figure 1. Pathological specimen: Cat kidney - Lymphosarcoma metastasis. North Carolina Cold-temperature technique.

Figure 2. Camel liver, parietal surface. North Carolina Cold-temperature technique.

Figure 3. Camel liver, visceral surface. North Carolina Cold-temperature technique.

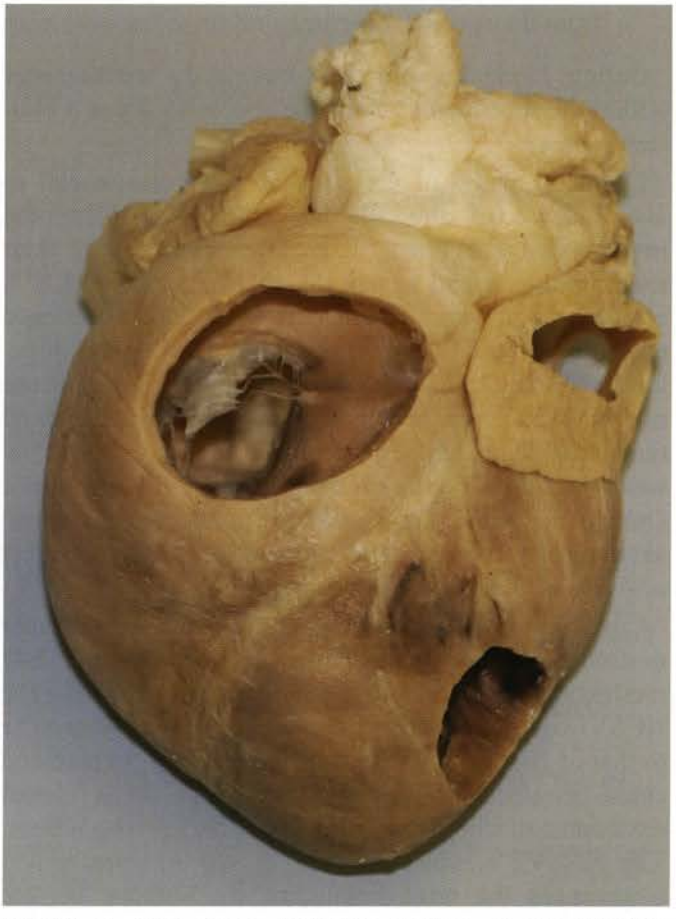

Figure 4. Dog heart auricular surface. North Carolina Ambient-temperature technique.

to get the pressure low enough for the solvent (acetone) to vaporize from the bottom specimens (Henry and Thompson, 1992; Henry, 2005b). The departing bubbles have to overcome the weight of the overlying polymer (von Horst, 2005). Therefore impregnation should be monitored closely.

The NCSX or XI polymer and NCSIII catalyst may also be used for impregnation at ambient-temperature. The protocol is similar to that of the "Biodur™ S10 ambient-temperature technique" (von Hagens, 1986). Impregnation can be completed in one week. The same precaution with the polymer impregnation-mixture must be adhered to. After impregnation, the mix must be returned to at least a minimum of a -25°C freezer to minimize thickening of the impregnation reaction-mix.

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Product distribution:

Silicone Inc., High Point, NC, USA. Distributed by: RW Henry, 1455 A. R. Davis Road, Seymour, TN 37865, USA. rhenry@utk.edu, Ph: (865) 982 - 4354.

Silicone Plastination of Biological Tissue: Cold-temperature Technique Additional Products

R.W. HENRY

Department of Comparative Medicine, College of Veterinary Medicine, University of Tennessee, Knoxville, TN, USA.

Correspondence to: Telephone: (865) 974 - 5822; *Fax:* (865) 974 - 5460; *E-mail:* rhenry@utk.edu

1. Preservation Specialties:

Product: Preservation Silicone Polymer 1 and 2; Tin Catalyst; Hardener/Crosslinker.

Distribution: Preservation Specialties, LLC; 4392 Otto Road; Charlotte, MI 48813; Phone: (517) 543-8213 selection #1; URL:*www.preservationspecialties.com; e-mail: pjo@preservationspecialties.com*

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2. Shin-Etsu Polymer:

Product: Shin-Etsu polymer KE-108, Catalyst CAT-108, RTV (room temperature vulcanizing) thinner.

Distribution: Shin-Etsu Chemical Co. Ltd., Silicone Division, 6-1, Ohtemachi 2-chome, Chiyoda-ku, Tokyo, 100-004 JAPAN. URL:http://www.shinetsu.co.jp/; *Fax:* (81) 3 3246 5362

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3. Sui/Hoffen:

Product: Silicone polymer - Hoffen R1; Catalyst - Hoffen R2; Hardener - Hoffen R6.

Distribution: Dalian Hoffen Bio-technique Co. Ltd, Room 301, No. 32, Lixian Street, Hi-Tech Zone, Dalian, China. URL:http://www.hoffen.com.cn; e-mail: wangjian599@hotmail.com; Fax: (86) 411 8475 4558

4. VisDocta Research Laboratories:

Product: Silicone polymer - VS-HS1; Catalyst - VS-HS1/D; Cross-linker - VS-HS1/G.

Distribution: VisDocta Research Laboratories, Dr. Tsabari Shahar, via Panoramica 2B, 25080 BS Tignale, Italy. visdocta@tin.it, http://www.visdocta.com/, Ph: (39) 3 65 761 000, Fax: (39) 3 65 761 900

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Silicone Plastination of Biological Tissue: Room-temperature Technique Dow™/Corcoran Technique and Products

A. RAOOF[1*], R.W. HENRY[2] and R.B. Reed[2]

[1]*Anatomy Division, Plastination Lab, University of Michigan Medical School, Ann Arbor, MI, USA.*
[2]*Department of Comparative Medicine, College of Veterinary Medicine, University of Tennessee, Knoxville, TN, USA.*

[*]*Correspondence to: Telephone:* (734) 615 2597; *Fax:* (734) 615 8191; *E-mail:* ameedr@umich.edu

Abstract: With the development of the superior Biodur™ S10/low-temperature plastination process nearly thirty years ago, it would not be unusual for similar silicone products to be developed as well as alternate techniques. VisDocta, Hoffen, Preservation Specialities and North Carolina low-temperature products use similar methods and chemicals as the Biodur™ S10 process. Most silicone products on the market could be used in either methodology, therefore nothing being truly unique. However, there is uniqueness with the Dow™/Corcoran/Room-temperature methodology. Dow changes the sequence in which basic plastination chemicals are added such that the impregnation-mixture (polymer plus cross-linker) is stable at room temperature. Recently, the North Carolina and Biodur™ chemicals have also been used successfully in this room-temperature format.

Key words: **plastination; silicone; PR10; PR14; Ct30; Ct32; Cr20; Cr22**

Introduction

A curable polymer replaces tissue fluid in silicone plastination. Silicone polymers and chemicals, other than the Biodur™ products, have been developed to carry out the silicone plastination process (Henry et al., 2001; Smodlaka et al., 2005a). The basic components are common products of the silicone industry and may be used to some degree interchangeably with caution (Henry et al., 2002). Principally, methods of placing the silicone into the biological specimen are the same: an intermediary solvent (acetone/methylene chloride) is removed by a decrease in pressure to extract the solvent (von Hagens, 1979a; 1979b; 1986; von Hagens et al., 1987; Henry and Nel, 1993). Hence a tissue void is produced and the polymer-mix is drawn into the cells. Each variant produces high quality durable plastinates.

The basic difference in methodology, excluding the polymers, is the sequence in which polymer, catalyst chain extender and cross-linker are combined. The basic cold S10 process combines the silicone polymer with the catalyst and chain extender to serve as the impregnation-mixture (von Hagens, 1986). This yields an unstable impregnation-mixture which commences to thicken upon mixing. Therefore, it is prudent to keep the mix in the cold to prevent premature thickening (increased viscosity). As noted in the earlier manuscript on S10, impregnation with this mix may be done also at ambient-temperature. However, the pot-life of the mix is greatly reduced. Uniquely, the Dow™/Corcoran/ Room-temperature method combines the silicone polymer with the cross-linker (Glover et al., 1998). This mix yields a stable impregnation-mix even when kept at room temperature. Therefore, there is no need to keep the impregnation-mix in the freezer for storage or for impregnation. Recent experience has demonstrated that

both the Biodur™ and the North Carolina products may also be used with this methodology.

Chemicals used in these "generic" silicone-plastination techniques are the same or at least similar to those used in the classic Biodur™ S10 Cold-temperature plastination process:

- Acetone,
- Silicone polymer,
- Cross-linker, to form a 3-D meshwork of the elongated silicone molecules by side to side linkage.
- Chain extender, to promote the silicone molecules to form longer-chain silicone molecules,
- Catalyst, to prepare the silicone molecules for elongation and cross-linkage,

The sequence of combination of these products is the difference in these two methodologies. The general steps of silicone plastination are described earlier in this volume. This manuscript will highlight the differences with respect to this variant process which occur during impregnation and curing of the plastinated specimens.

Materials and methods

The basic steps of plastination are used for each plastination technique:

Steps 1 and 2 (Specimen preparation and *Dehydration)* are the same for all plastination methods, even with the Dow methodology. Please refer to: "The S10/15 Plastination Cold-temperature Technique" for this information. Adequate specimen preparation and thorough cold dehydration are essentials for producing good plastinates.

The more common Dow™ products for silicone plastination are:

PR10 and PR14 (lower viscosity): Silicone polymer
Ct30 and Ct32: Catalyst and chain extender, premix
Cr20 and Cr22: Cross-linker

Step 3. Forced Impregnation:

Replacing the volatile solvent (acetone) in a biological specimen with a curable polymer. For this to occur, the same criteria as for the classic Biodur™ S10 plastination process must be met, including thorough cold dehydration.

Impregnation equipment: Similar to the Biodur™ S10 requirements - Vacuum chamber and pump (oil or dry); Specimen basket; Vacuum gauge; Bennert manometer; Needle valves. One exception: no deep freezer is needed for impregnation or storage of impregnation-mixture. However for dehydration, freezers are highly recommended.

Preparing reaction-mixture: Dow™ PR10 or PR14 polymer is mixed with Cr20 or Cr22 (Cross-linker and Chain extender) at **100:10** to prepare the reaction-mixture. Thorough stirring is recommended. One of the two main differences is how the reaction-mixture is to be handled. The reaction-mixture is stored and used at room temperature (Glover et al., 1998; Glover, 2004; Raoof, 2004). It is stable (does not become viscous) for at least ten years at room temperature.

Adjusting vacuum: The speed for lowering of pressure in the vacuum chamber is very fast when compared to the Biodur™ S10 Cold-temperature Technique. The viscosity of the PR10 polymer is 20% less than the viscosity of S10 polymer. The impregnation-mixture is not reactive, as long as it is not exposed to catalyst. Therefore, the polymer-mix remains fluid. As well, it is used at room temperature so there is no increase of viscosity from placing the mixture in a cold environment. When monitoring bubble formation, a rapid boil is the standard. If in doubt, go slower, to prevent incomplete impregnation with resulting shrinkage. Pressure is decreased by closing the air intake valves.

Impregnation regimen:

Day 1: The dehydrated and degreased specimens are removed from the solvent (acetone), excess solvent is drained and the dehydrated, solvent-filled specimens are placed in the room-temperature polymer impregnation-mixture. Submerge the specimens immediately to prevent solvent evaporation from their surface and hence, surface drying. Close the port of the vacuum chamber with the glass and let the specimens accommodate/equilibrate over night.

Day 2: Warm the vacuum pump a few minutes. Seal the vacuum chamber and lower pressure (around ½ atmosphere) until bubble formation becomes rapid. Stabilize pressure at the level which maintains a rapid boil. Monitor pressure and decrease the pressure as needed to maintain a rapid boil.

Day 3: Continue to monitor bubble formation (solvent extraction) maintaining a rapid boil by decreasing pressure (incrementally close needle valves) as needed.

Day 4a: Small specimens will likely finish impregnation as noted by cessation of bubble formation. Turn off pump and bring specimens to atmospheric pressure. Proceed to Step 4 - "Curing/hardening".

Day 4b: Large specimens or a large quantity of specimens. Continue to monitor solvent extraction (watch bubble formation) and maintain a rapid boil by incrementally closing the needle valves.

Day 5 or plus X: Continue to monitor solvent extraction (watch bubble formation) and maintain a rapid boil. When impregnation is completed, as noted by cessation of bubble formation, turn off pump and bring specimens to atmospheric pressure.

Rule: If bubbles are actively rising to the top of the polymer and bursting, impregnation is not finished! Impregnation will be complete when needle valves are closed and no more rapid bubbling is noted and when pressure reaches <5mmHg. Nearly complete evacuation of acetone/solvent is necessary to avoid incomplete impregnation of the specimen with the polymer-mix which may lead to shrinkage.

Day 1	Load specimens and allow equilibration over night.
Day 2	Start pump, Decrease pressure until rapid boil is produced (around 28cm/13in Hg). Maintain rapid boil - by decreasing pressure (close needle valves incrementally).
Day 3	Maintain rapid boil by decreasing pressure (close needle valve incrementally).
Day 4a	Small specimens: Maintain rapid boil until boiling ceases. When boiling ceases, turn off pump, return to atmosphere and proceed to Step 4 - "Curing".
Day 4b	Large specimens: Maintain rapid boil - Decrease pressure incrementally as necessary.
Day 5 + X	Maintain rapid boil until boiling ceases. When boiling ceases, turn off pump, return to atmosphere and proceed to Step 4 - Curing.

Table 1. Impregnation schedule for PR10 and PR14 - Dow room-temperature technique.

Specimen removal and drainage of polymer-mix:
Follow S10 protocol. Since keeping the reaction-mix cold is not necessary, specimens are drained for an extended period directly into the room-temperature plastination chamber.

Step 4. Curing (Hardening or Cross-linking) of the impregnation-mix within the specimen to make it dry:
Equipment for curing:

- Absorbent paper: To wipe excess polymer-mix from the specimen.
- Paint brush or Mist bottle: To apply the Ct30 or Ct32 to the specimen.
- Foil (plastic wrap): To seal the specimen in an air-tight environment and keep the Catalyst next to the impregnated specimen.

Curing/Cross-linking is a two-step process:

a. Drainage of the excess silicone-mix. Drain excess polymer-mix from the specimens into the plastination chamber.

b. Catalyzing, cross-linking of the silicone polymer molecules. This reaction occurs because as the catalyst (Ct30 or Ct32) is applied to the impregnated specimen, it prepares the PR10 or PR14 silicone molecules in the impregnation-mix to react with the cross-linker (Cr20 or Cr22) in the impregnated specimen. Catalyst is applied to the specimen surface via a mist bottle or brush. Then the catalyst covered specimen is wrapped in foil. The next day foil is removed and the specimen checked to see if cured (dry). If not completely cured, the specimen surface is wiped and catalyst is applied to the specimen. The specimen is rewrapped with foil. The following day the specimen is checked for complete curing. If curing is complete, the specimen is ready for use. If curing is not complete, more catalyst is applied and the specimen is wrapped again in foil, etc.

Day 1	Bring specimens to atmospheric pressure and allow to drain into the plastination chamber.
Day 2	Place specimens on absorbent toweling. Allow excess polymer-mix to drain. Wipe excess polymer from the surface. Blow polymer-mix from hollow organs.
Day 3	Wipe excess polymer-mix from surface of specimen. Apply Ct30 or Ct32 to specimens and wrap in plastic wrap.
Day 4	Unwrap specimen and examine curing rate. If specimen is wet, apply more Ct30 or Ct32 and rewrap with foil. If curing is complete, specimen is ready to use.
Day 5	Unwrap specimen and examine curing rate. If necessary apply more Ct30 or Ct32, etc. If curing is complete, specimen is ready to use.
Day 6	Use specimen as desired.

Table 2. Curing schedule for PR10 and PR14.

Results

The PR10/PR14 specimens are durable and some flexibility is exhibited by thinner specimens (Glover et al., 1998; Glover, 2004). The specimens are dry and odorless. They are not models but the actual specimens. Numerous specimens and whole cadavers have been successfully plastinated using the room-temperature technique.

Discussion

Plastinated specimens are the real specimen and not a model (Figs. 1-6). All specimens are free of offensive odor and dry (Latorre et al., 2001). One feature of the finished product is a less than transparent surface which

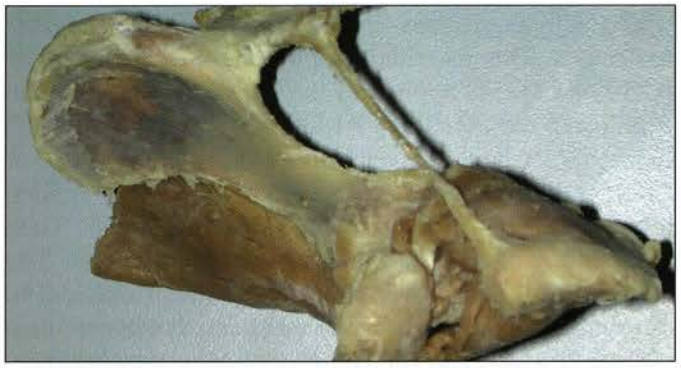

Figure 1. Lateral view of left canine coxal region - Dow Room-temperature technique.

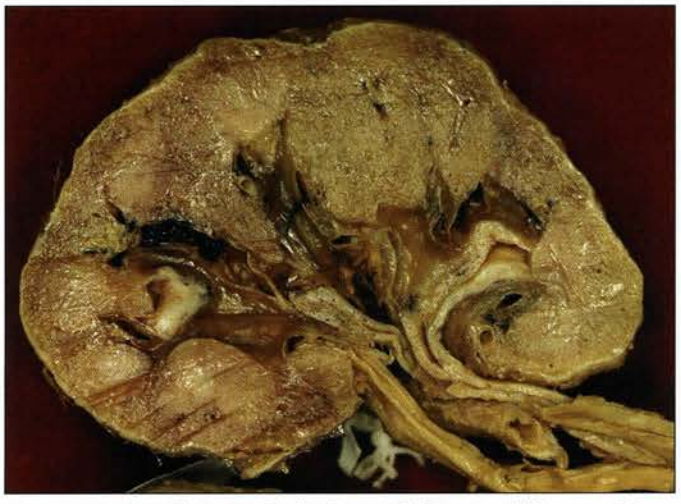

Figure 2. Median sagittal view of human kidney - Dow Room-temperature technique.

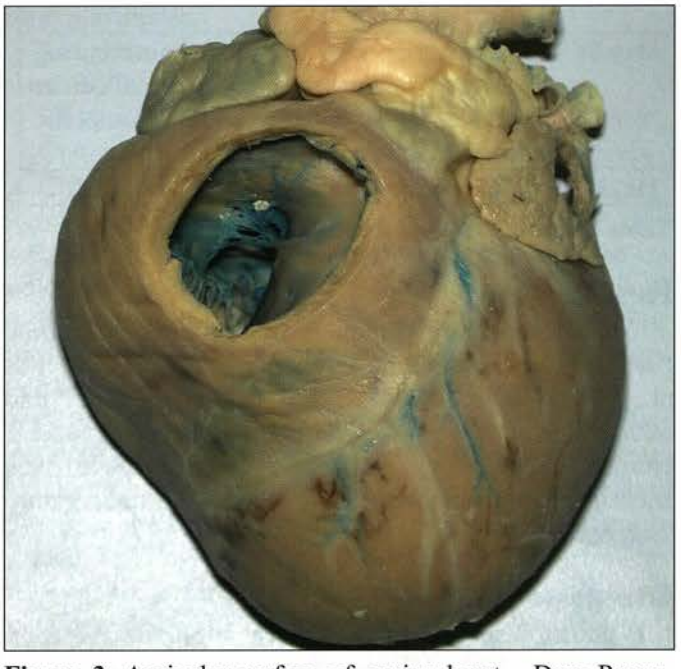

Figure 3. Auricular surface of canine heart - Dow Room-temperature technique.

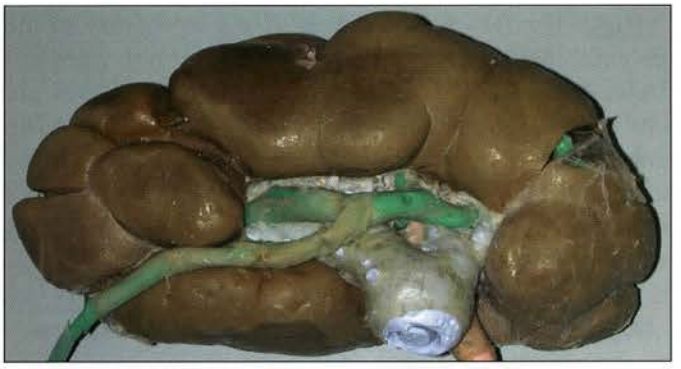

Figure 4. Bovine kidney - Dow Room-temperature technique.

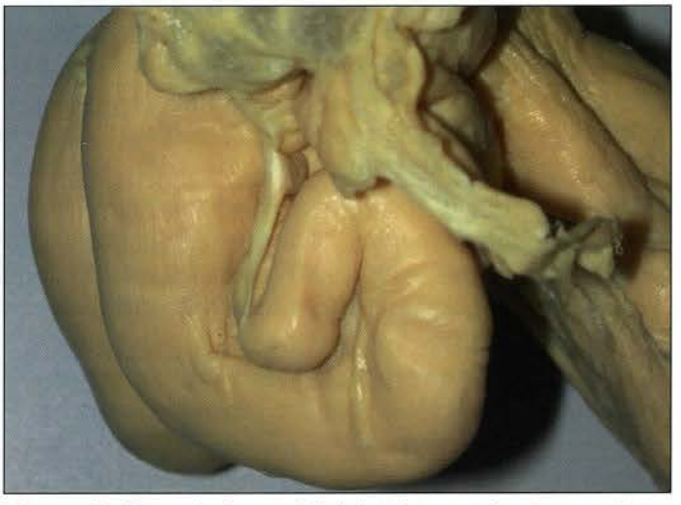

Figure 5. Lateral view of left bovine uterine horn - Dow Room-temperature technique.

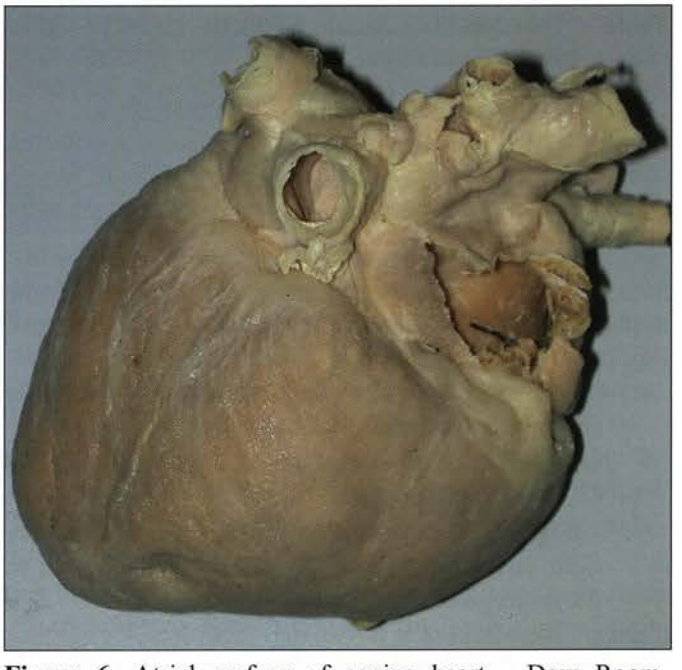

Figure 6. Atrial surface of canine heart - Dow Room-temperature technique

conceals intricate surface cellular detail (Henry et al., 2004; Smodlaka et al., 2005a; 2005b). Room temperature specimens may be used as teaching aids both in the class room and the clinical setting (Latorre et al., 2001; Raoof et al., 2006). They may be compiled and used as a library of specimens for normal, exotic and pathological anatomy (Henry, 2005) and in research (Raoof, 2001).

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Product distribution:

Corcoran Laboratories, Inc., Daniel Corcoran, Corcoran Laboratories, Inc. P. O. Box 3251, Traverse City, MI 49685-3251, Tel. (231) 922-8044.

Silicones Inc., High Point, NC, USA. Distributed by: RW Henry, 1455 A. R. Davis Road, Seymour, TN, 37865, USA. rhenry@utk.edu, Ph: (865) 982 - 4354.

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Silicone Plastination of Biological Tissue: Room-temperature Technique North Carolina Technique and Products

R.W. HENRY

Department of Comparative Medicine, College of Veterinary Medicine, University of Tennessee, Knoxville, TN, USA.

Correspondence to: Telephone: (865) 974 5822; *Fax:* (865) 974 5822; *E-mail:* rhenry@utk.edu

Abstract: The development of the Biodur™ S10 low-temperature plastination process thirty years ago has sparked development of similar methodologies and products. The Dow/Room temperature-process used silicone chemicals similar to the Biodur™ products. However, Dow changed the order in which the basic Biodur™ S10 plastination chemicals were combined. This combination renders the impregnation-mix non-reactive and hence deep freezers are not needed or used for impregnation. Recently, both the North Carolina and Biodur™ chemicals both have been shown to be compatible for use as a room-temperature format. The North Carolina Room-temperature technique combines the cross-linker and polymer for impregnation, but uses chain extender prior to using the catalyst after impregnation.

Key words: **plastination; silicone; polymer; NCSX; NCSXI; NCSIII; NCSV; NCSVI**

Introduction

Silicone plastination replaces tissue fluid with a curable polymer. Besides the Biodur™ products, other silicone polymers and chemicals, all well-known in the silicone industry (Henry et al., 2002a), have been developed for use in the plastination process (Henry et al., 2002b). Forced impregnation of silicone polymer into biological specimens is the common thread for all of the generic plastination products. Impregnation uses the same intermediary solvent (acetone) along with a decrease in pressure to extract the acetone (von Hagens, 1979a; 1979b; 1986; von Hagens et al., 1987; Henry, 2004). This resultant tissue void allows the impregnation-mix to be drawn into the specimen. Each generic product produces a durable, high quality plastinated specimen.

Chemicals used in the various "alternate" silicone-plastination processes need to be similar to the Biodur™ S10 plastination technique:

- Acetone
- Methylene chloride
- Silicone polymer
- Cross-linker, enables side to side linkage and formation of a 3-D meshwork to the elongated silicone molecules
- Chain extender, promotes the silicone molecules in formation of longer-chain silicone molecules
- Catalyst, prepares the silicone molecules for elongation and cross-linking

The general steps of silicone plastination are described earlier in this volume. This manuscript will highlight the differences with respect to this alternate process which occurs with room-temperature impregnation and curing of the plastinated specimens.

Materials and methods

The basic steps of plastination are utilized for each plastination process:

Specimen preparation dehydration and defatting

Specimen preparation, dehydration and defatting are the same for all plastination methods. Please refer to: "The S10/15 cold-temperature plastination technique" for that information. As well, refer to the "Dow room-temperature technique" for additional information.

North Carolina products for silicone plastination:

NCSX (lowest viscosity): silicone polymer
NCSXI (low viscosity): silicone polymer
NCSIII: catalyst
NCSV: chain extender
NCSVI: cross-linker

Forced impregnation

Replacing the volatile solvent (acetone or methylene chloride) in a specimen with a curable polymer. For this to happen, the products must meet the same conditions as for the Biodur™ S10 plastination process.

Impregnation equipment: Similar to Biodur™ S10 requirements [vacuum pump (oil preferred) and chamber with see through port and specimen basket, vacuum gauge, manometer, needle valves]. However, no deep freezer is required or used for impregnation. Deep freezers are necessary and strongly recommended for dehydration.

Preparing the impregnation-mixture: NCSX or NCSXI polymer is mixed with NCSVI (Cross-linker) at **100:8** to prepare the impregnation-mixture and stirred thoroughly. The main difference in this room temperature methodology is handling of the resultant reaction-mixture. This mixture is stable (does not become viscous) when stored and/or used at room temperature for an indefinite period of time as is the case with other generic room-temperature polymers.

Adjusting the vacuum: Speed of lowering the pressure in the vacuum chamber is fast when compared to the Biodur™ S10 Cold-temperature Technique. The NCSX and NCSXI polymers have a much lower viscosity than Biodur™ S10 or Dow™ PR10. The impregnation-mixture is not reactive, provided it is not exposed to catalyst. There-fore the polymer-mix remains fluid at impregnation (room) temperatures As well, since there is no increase of viscosity which results from placement in a cold environment. When monitoring bubble formation, a rapid boil is recommended. Remember the plastination principal, if in doubt, decrease pressure slower, to prevent incomplete impregnation which can result in shrinkage.

Impregnation regimen

Day 1: The dehydrated and degreased specimens are removed from the solvent (acetone or methylene chloride). Excess solvent is drained and the dehydrated, solvent-filled specimens are placed in the room-temperature polymer impregnation-mixture. Submerge the specimens immediately to prevent solvent evaporation from their surface and hence, drying. The port (glass) on the vacuum chamber is closed and the specimens allowed to accommodate/ equilibrate in the polymer-mix overnight.

Day 2: The vacuum pump is warmed briefly. Close the needle valves and lower pressure (apply vacuum) to seal the chamber. Lower the pressure until bubble formation becomes rapid (decrease to around 30-25cm/13.5-13in Hg pressure). When a rapid boil is achieved, maintain this pressure by opening the vacuum adjustment valves slowly until pressure is stabilized. The vapor pressure of acetone at room temperature (+25°C) is 22cm/10in Hg and MeCl is 43cm/17in Hg. Continue to monitor the pressure and decrease the pressure as needed to maintain a rapid boil by closing the needle valves incrementally. Likely adjustment will be every one half hour for the first three or four hours.

Day 3: Continue to monitor bubble production (solvent extraction) and keep a rapid boil by decreasing pressure (closing the valves) as needed.

Day 4a: Small specimens - Impregnation will be complete as noted by cessation or reduction in number of 1-2cm size bubbles forming and/or reaching <1cm Hg of pressure. Note: When pressure goes below 4 or 5 mm Hg, larger bubbles will rise and burst if the chamber is tilted or shaken. These are not likely the solvent (acetone) but likely water vapor or the cross-linker, NCSVI vaporizing. Vacuum should be turned off when this occurs. Turn off pump and bring specimens to atmospheric pressure. Allow the impregnated specimens to sit in the polymer-mix over night at atmospheric pressure.

Day 5: Proceed to Step 4 - "Curing".

Day 4b: Large specimens or a large quantity of specimens. Continue to monitor solvent extraction by watching bubble formation and by reading and adjusting pressure to maintain a rapid boil (extraction of the solvent).

Day 5 or plus X: Continue to monitor solvent extraction (watch bubble formation) and maintain a rapid boil. If impregnation is completed, as noted by cessation or reduction of 1-2 cm size bubble formation and/or reaching 5mm Hg of pressure, turn off pump and bring specimens to atmospheric pressure (see note in Day 4a). Proceed to Step 4 - "Curing".

Rule: If 1-2 cm bubbles are actively rising to the top of the polymer and bursting, impregnation is not finished! Impregnation will be complete when both needle valves are closed, pressure has reached <5mm Hg and/or 1-2cm diameter bubble production is greatly diminished. Note, once the 1-2cm bubbles have subsided, larger 4-5

cm bubbles will explode to the top if you shake the vacuum chamber. These are likely not acetone bubbles but either water vapor or cross-linker (NCSVI) vaporizing. Nearly complete evacuation of acetone/solvent is necessary to avoid incomplete impregnation of the specimen with the polymer-mix which may lead to shrinkage.

Day 1	Load specimens and allow to equilibrate over night.
Day 2	Start pump: Decrease pressure until rapid boil is produced (around 30 to 25cm/13.5 to 13in Hg pressure). Maintain rapid boil - Decrease pressure (incrementally, close needle valves) as needed.
Day 3	Maintain rapid boil - Decrease pressure (close needle valve incrementally).
Day 4a	Small specimens: Maintain rapid boil until boiling ceases (4-5mm Hg) or when 1-2cm bubbles cease to form, turn off pump, return to atmosphere, allow specimens to equilibrate overnight and proceed to Step 4: Curing, the next day.
Day 4b	Large specimens: Maintain rapid boil – Decrease pressure as necessary.
Day 5 + X	Maintain rapid boil until 1-2cm bubbles cease and/or 5mm Hg pressure is reached. Turn off pump, return to atmosphere and proceed to Step 4 - Curing.

Table 1: Impregnation schedule for NCSX or NCSXI (polymer)/NCSVI (cross-linker). North Carolina room-temperature silicone technique.

Specimen removal and drainage of surface polymer impregnation-mix: Follow the S10 protocol. Since the impregnation-mixture is stable, drain specimens into the room-temperature plastination chamber.

Curing (hardening or cross-linking)

Equipment for curing:

- Absorbent paper to wipe excess polymer-mix from the specimen.
- Closed environment for NCSV application via vaporization.
- Gloved finger, paint brush or mist bottle to apply NCSIII (cross-linker) to the specimen. Do not reintroduce gloved finger or brush into stock reservoir of catalyst if specimen has been touched. The stock solution will be contaminated with cross-linker and/or polymer and at first become viscous and eventually solidify.
- Foil (plastic wrap) to seal the specimen in an air-tight environment and keep the Catalyst (NCSIII) next to the impregnated specimen.
- NCSV - chain extender and NCSIII - catalyst

Curing of the impregnation-mixture within the specimen is a three-step process:

Drain: Drain the excess impregnation polymer-mix from the specimens.

Chain elongation: Chain elongation of the silicone polymer molecules by end to end alignment of the molecules. Chain elongation occurs as the NCSV vapor is applied to the surface of the specimen. NCSV is vaporized in an enclosed chamber by using an aquarium pump or ventilator for a few minutes once a day for one to three days. This process is similar to the cross-linking application in the Biodur™ S10 Cold- or Ambient-temperature technique.

Day 1	Bring specimens to atmospheric pressure and allow to drain into chamber.
Day 2	Empty polymer-mix from hollow organs. Place specimens on absorbent toweling to drain polymer-mix. Wipe excess polymer from the surface.
Days 3 and 4	Manicure specimen surface, dilate and/or position anatomically. Chain extend: Vaporize the NCSV in an enclosed environment.
Day 5	Wipe excess polymer-mix from surface of specimens. Apply NCSIII to specimen surface and wrap in foil/plastic wrap.
Day 6	Unwrap specimen and examine curing rate. If necessary, apply more NCSIII and rewrap with foil. If the curing is complete, specimen is ready to use.
Day 7	Unwrap specimen and examine curing rate. If necessary, apply more NCSIII. If curing is complete, specimen is ready to use.
Day 8	Use specimen as desired.

Table 2. Curing schedule for NCSX or NCSXI. North Carolina room-temperature silicone technique.

Catalyzing and cross-linking: Catalyzing and cross-linking of the silicone polymer molecules. This reaction occurs when the catalyst (NCSIII) is applied to the surface of the impregnated specimen. It enables the NCSX or XI molecules in the impregnation-mix to react with the NCSVI which is in the impregnated specimen. Cross-linkage changes the polymer from a liquid to a solid. Mist or wipe NCSIII onto the surface of the specimen, wrap specimen with foil and leave over night. Wrapping in foil (plastic wrap) is necessary to facilitate cross-linking.

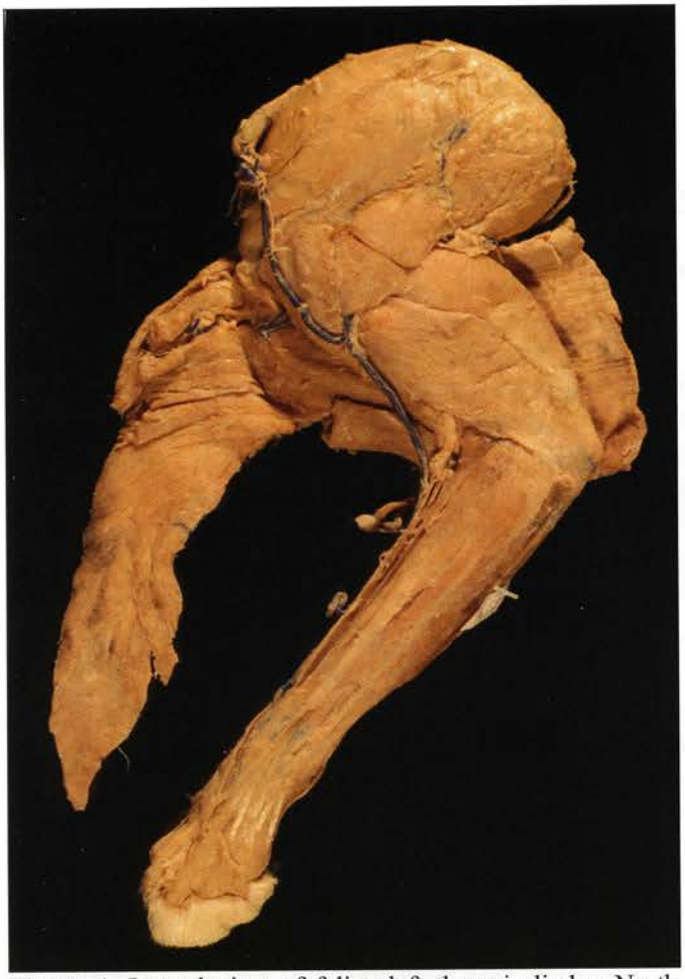

Figure 1. Lateral view of feline left thoracic limb - North Carolina Room-temperature silicone technique.

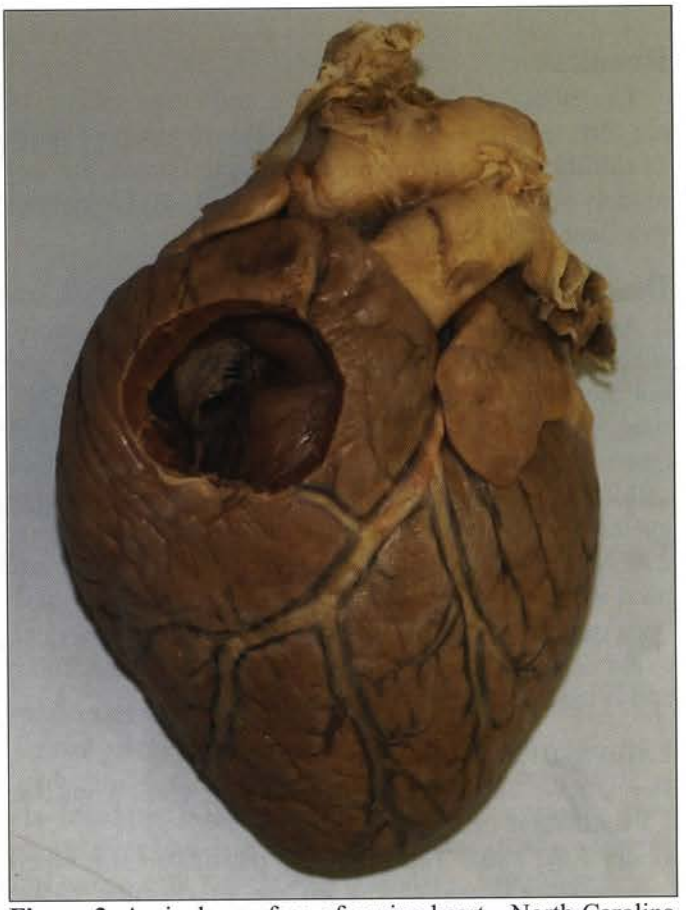

Figure 3. Auricular surface of canine heart - North Carolina Room-temperature silicone technique.

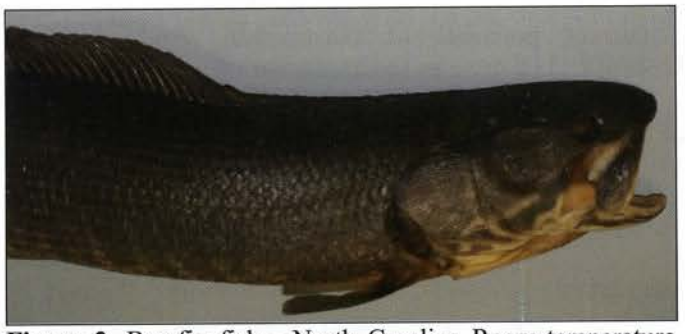

Figure 2. Bowfin fish - North Carolina Room-temperature silicone technique.

Figure 4. Dorsal view of prosected equine brain stem - North Carolina Room-temperature silicone technique.

Results

The NCSX or XI impregnated and cured specimens are dry and durable. Thin specimens exhibit some flexibility. The room-temperature plastinates are not models but real specimens (Figs. 1, 2, 3, 4). They make excellent teaching and public relation aids.

Discussion

As with all plastination processes, the room-temperature plastination produces real specimens and not models. The specimens are dry and odorless. Only time will reveal if the NCSX develops a less than transparent surface which conceals intricate surface cellular detail as seen with the Dow™/Corcoran chemicals (Henry et al., 2004; Smodlaka et al., 2005a; 2005b). Room-temperature technique specimens are used as teaching aids (Latorre et al., 2001). They have been used to compile a library of specimens for normal, exotic (Fig. 2) and pathological anatomy (Henry, 2005) and is useful in research (Raoof, 2001).

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Silicones Inc., High Point, NC, USA. Distributed by: RW Henry, 1455 A. R. Davis Road, Seymour, TN, 37865, USA. rhenry@utk.edu, Ph: (865) 982 - 4354.

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Epoxy Plastination of Biological Tissue: E12 Technique

M-C. SORA[1*] and P. COOK[2]

[1]*Plastination Laboratory, Centre for Anatomy and Cell Biology, Plastination and Topographic Anatomy, The Medical University of Vienna, Währingerstr. 13/ 3, A-1090 Wien, Austria.*
[2]*Human Anatomy Laboratory, Department of Anatomy with Radiology, School of Medicine, University of Auckland, New Zealand.*

[*]*Correspondence to: Telephone:* 43-1-4277-611-50; *Fax:* 43-1-4277-611-70; *E-mail:* mircea-constantin.sora@meduniwien.ac.at

Abstract: Since its development 30 years ago, plastination has become the best method for preservation of perishable biological human and animal tissue. In recent years, plastination has been utilized in the study of different anatomical and clinical questions. Using the Biodur™ E12 technique, transparent tissue slices are produced which exhibit detailed structures in their normal anatomical position, especially regarding muscular, vascular and interstitial tissue. The Biodur™ E12 method of plastination is used to create 2-5mm transparent slices. Many modern diagnostic techniques including radiography, computed tomography, magnetic resonance imaging and ultra sound underscore the importance of understanding serial sectional anatomy of the body. The Biodur™ E12 plastination method allows production of precise transparent sectional preparations of superb visual clarity. These sections allow visualization of gross structures as well as submacroscopic structures with the naked eye. Further study of E12 sections at the histological level is also possible with the use of light microscopy and electronic video equipment.

Key words: **plastination; epoxy; E12; E1; AE30; AE10; slices; body**

Introduction

Today's modern diagnostic imaging techniques (computed tomography, magnetic resonance imaging, and ultrasound) underscore the importance of understanding sectional anatomy of the human and animal body. The purpose of E12 sheet plastination is to preserve 2-5mm slices of tissue for examination/study by replacing all tissue fluid and a significant amount of fat with a curable resin. The Biodur™ E12 plastination method provides accurate, precise and semi-transparent sectional preparations which offer superb visual clarity of gross structures down to a submacroscopic level viewable with the naked eye.

Body slices (2-5mm thick) are produced using a flat chamber or a sandwich technique. The E12 method is named for the epoxy resin Biodur™ E12 used for this process. As in other plastination methods, water and some lipid are removed from the slices and replaced with the curable epoxy resin-mix. Chemicals used in epoxy-plastination include acetone, epoxy resin, epoxy hardeners, and epoxy plasticiser.

Two techniques for producing E12 impregnated slices are: The sandwich technique and The flat chamber technique. As with other methods of plastination, the standard Biodur™ E12 technique may be modified by omitting or changing procedural steps. However, in order to obtain the best results, the standard description of the Biodur™ E12 technique should be followed.

Teaching with clinical correlation as well as having powerful research potential are the hallmarks of this exciting technique. This standard protocol describes plastination of sliced body parts via the E12 method.

Materials and methods

The standard steps of Biodur™ E12 plastination are specimen preparation, cold dehydration, degreasing, impregnation and curing (von Hagens, 1986; Fasel et al., 1988; Weber and Henry, 1993; Cook, 1996; Cook and Ali-Ali, 1997; An and Zhang, 1999; Sora et al., 2002; Sora et al., 2004).

The general protocol for production of E12 - epoxy (2-3mm) slices is found in table 1 for the frozen specimen (-75°C).

Specimen preparation

The desired specimen is selected and may or may not be fixed with formalin. Fixation is not necessary for production of semi-transparent body slices using E12 plastination. In general it is desirable that tissue be fixed to prevent any potential biohazard risk which may accompany handling and sawing of human or animal tissue. The disadvantages associated with tissue fixation are a marked loss of tissue color and an increase in the freezing point of the specimen. The specimen should be positioned in proper anatomical position prior to fixation.

It is important to freeze the portion of the body chosen for sectioning at -75°C, especially if the tissue was fixed. If unfixed tissue is being used, the specimen should be placed in proper anatomical position prior to freezing. Shock freezing at -75°C is necessary to reduce the formation of ice crystals. Common freezers which achieve -25°C are not ideal because ice crystals may develop in the tissue during freezing and the harder the specimen is frozen the better slicing will be. Pre-cooling of specimens to +5°C overnight prior to freezing can help prevent ice formation. Specimens need to be frozen uniformly. Therefore, keep the specimen in the deep freezer for five days to assure complete freezing. Large specimens should be subdivided for ease of handling and to avoid defrosting of the specimen during slicing. For example, a torso should be divided into several parts prior to producing slices from each part. Hairy specimens should have the hair clipped prior to sectioning.

Sawing: Body slices (large or small) can be sliced on a butcher band saw. Smaller specimens are technically easier to saw. The band saw should have a guide stop and be constructed of stainless steel for easy cleaning and maintenance. A saw with a cooled guide stop is beneficial but not essential. Cooling the guide stop for at least two hours at -25°C will help prevent thawing of the slices and the specimen. The ideal slice thickness is 2-3mm which assures a transparency of the connective tissue in the finished specimen. Sections greater than 3mm in thickness are typically too dense and contain many superimposed structures which substantially diminish clarity. For best results, band saw speed should be 20-35m/sec. Slices may be cleaned of sawdust for immediate processing or be stored between polyethylene foil in a deep freezer for later use.

Saw dust removal: Freshly sawn slices are usually covered with a thick layer of tissue shavings on each side (Fig. 1). These tissue particles must be removed because they will appear as artefacts on the finished product. This saw dust may be removed by scraping the slices while frozen, either before or after immersion in cold acetone. Flushing the surface with a small cool stream of tap water while frozen or brushing them in the last dehydration bath at room temperature or any combination of the aforementioned methods as is deemed suitable by the investigator are acceptable. Any of these methods have some disadvantages; therefore there is no ideal method. The recommended method is to scrape the dust off using a scalpel or sharp broad knife while frozen in the freezer. To do this, the slice is placed on a cold 10mm metal plate, which has been cooled for several hours at -25°C. Using the edge of a length of Perspex (Plexiglas) to scrap the frozen tissue may be a less hazardous method than using a scalpel since it is passed gently over the surface in a single sweeping movement. Also, the slice may be submerged in -25°C acetone to prevent defrosting of the slice. The dust can then be scraped from the surface of slice while they are frozen and submerged. Brushing the slices in the last dehydration bath is more difficult and not easily done if slices contain intestinal loops or other small pieces. If a stream of water is used, it must be done quickly to avoid thawing of the slice. The cleaned slices are then placed on acetone resistant grids and submerged in cold (-25°C) acetone.

Dehydration and degreasing

The standard dehydration procedure for plastination is freeze substitution. During freeze substitution, specimens are submerged in cold (-25°C) acetone (technical grade) and immediately freeze upon immersion thereby stabilizing the specimen's shape instantly. This method saves time and labor when compared with ethanol dehydration. In addition, shrinkage is minimal when cold acetone is used (less than 7%). Alcohol shrinkage is two to four times greater.

For all plastination methods, the use of acetone is ideal as it serves as the dehydration agent, degreasing agent and intermediary solvent and readily mixes with

all the resins and polymers used for plastination.

To aid dehydration, fly screen (mesh) (Fig. 2) is placed between the slices. To aid transfer of slices into succeeding acetone baths, a metal or plastic grid is placed at each end of the stack and after every fifth slice for stability. The correct succession is: grid - screen - tissue slice - screen - tissue slice - screen - tissue slice etc. This arrangement permits acetone to circulate between slices and assures thorough dehydration and ease of handling. Once fifteen slices are stacked using this sequence, the slices are tied together as a bundle using string or stacked in a basket and submerged in cold acetone (Fig. 3). If many slices are prepared, baskets to contain the slice bundles allow for easier handling and changing of acetone baths.

Preferably, saw dust free slices are immersed in the first dehydration bath. If >90% acetone is available from previous dehydration baths, it may be used for this first bath. If less that 100% acetone is not available, use fresh technical grade acetone (99-100%). A recommended tissue to fluid ratio is at least 1:5-10. The basket containing the tissue slices within the screen/grids should be introduced into the acetone at an angle. This prevents trapping air bubbles within the sections, mesh and grids and permits acetone to flow evenly upwards and across the tissue surface at an oblique angle. The basket should be moved from side to side as an additional measure to remove trapped air bubbles. The submerged slices need to be adequately covered with acetone.

Likely three acetone baths will be necessary. The thin slices and separation by screen will allow the slices to dehydrate in seven to nine days. After three days in the first bath, acetone is changed for new/fresh acetone, #2 bath (Fig. 4). Specimens should be transferred quickly from bath to bath and not allowed to dry. Dried areas will show up later as white patches of shrinkage in the finished product. After three days in bath #2, the specimens are transferred into bath #3. It is good to check acetone purity of bath #2. This will assure complete dehydration or if bath #3 is necessary. The acetone should be stirred and make sure acetone temperature is the same as the calibration temperature of the acetonometer. Most acetonometers are calibrated at +15, +20, or -10°C. To obtain an accurate reading, acetone must be brought to the calibrated temperature before reading the purity. If acetone concentration is at least 98.5%, dehydration is complete and bath 3 is not necessary. If acetone purity is <98%, the specimens need to be transferred into bath #3. The acetone concentration should be checked at the end of bath #3 (day 11) and conclusions on the extent of dehydration are determined. If dehydrated thoroughly, the slices are ready for degreasing.

The last dehydration bath, with a final concentration of at least 98.5%, is brought to room temperature to start the degreasing process. Degreasing is a vital step for production of good slices using E12 methodology. If specimens do not contain much fat, acetone is usually adequate to assure enough lipid removal. Lipid extraction from the specimen is essential to create transparency and good definition between structures of the E12 impregnated slice. When the acetone has turned yellow, the specimens must be transferred through one or two more room temperature acetone baths. Usually a minimum of two weeks and two or three acetone room temperature changes are needed. However, many slices contain high amounts of lipid and need a much stronger lipid remover. Hence after dehydration bath #3, these specimens are transferred into methylene chloride [dichloromethane (MeCl)]. MeCl is the ideal fluid for degreasing. MeCl is a hazardous substance and must be handled and kept in a ventilated hood. There is no method to measure the fat concentration of the used MeCl or acetone. Therefore, the color of the acetone/MeCl baths must be monitored for color change to yellow. When the color becomes an intense yellow, the bath is changed. MeCl degreasing takes less than one week, with one mid-week change to new MeCl. To assure adequate degreasing, it is necessary to visually inspect the quality of the degreased slices.

Forced Impregnation

Forced impregnation is the central and very important process in plastination. During impregnation, the solvent is extracted from the cellular and interstitial space of the slice and is replaced with the resin impregnation-mix. The epoxy resin and hardeners are mixed as follows to prepare the E12 *impregnation-mixture*: E12 - 95pbw, AE30 - 5pbw, AE10 - 20pbw, E1 - 26pbw. The epoxy reaction-mixture has a relatively short pot life which cannot be extended by freezing as with the silicone reaction-mixture. At freezing temperatures, E12 crystallizes and becomes too viscous for exchange in the cold. Crystallization of the E12 resin may also occur during routine storage prior to adding hardener. Storage crystallization can be reversed by warming the resin to 80°C. However, it must cool to room temperature before the hardener is added.

Only enough volume of reaction-mixture is prepared to adequately cover the intended volume of slices for impregnation. Larger quantities of resin reaction-mixture cure faster. Therefore, the specimen impregnation reservoir size should be planned to minimize an excess space around the perimeter of the stack of the slices. The reaction-mixture is placed in the

impregnation vat in the room temperature or +5°C vacuum kettle. The dehydrated and degreased slices are transferred from the degreasing bath (MeCl or Acetone) into the impregnation bath. The bundle of stacked slices and screens are rapidly submerged (to avoid drying of the slices) into the impregnation bath. The stack of slices tends to float and must be weighted down or held down by the basket lid. If MeCl is used, transfer should be done in a ventilated hood. A safety level of resin of at least 3-5cm over the top of the slices is necessary. Lining the vacuum chamber and covering the impregnation reservoir with plastic foil/wrap ensures easy clean up of the splashing epoxy (Fig. 5). A thermometer is placed in the vacuum kettle (Fig. 5).

The vacuum pump is turned on, prior to applying vacuum for 10 minutes, to allow the pump to warm to working temperature. The box containing the tissue slices and the E12 impregnation-mixture is placed in the vacuum chamber, at +5°C or room temperature (RT). Once the vacuum kettle has been prepared, vacuum is applied and the kettle is sealed. The rate of lowering pressure and hence extraction of solvent is rapid. Pressure is continuously reduced, but slowly, starting at atmosphere (76cm Hg) down to 2mm Hg over nearly two days. The volatile intermedium has a low vapor pressure and the resin-mix a high vapor pressure. Therefore as pressure is lowered and the solvent boiling point is reached, the volatile intermedium vaporizes and bubbles out of the specimen into the resin and is extracted out through the pump exhaust. However, in the first two hours as pressure is decreased frequently, only air is evacuated until the boiling point of the solvent is reached. At +5°C, 8.5cm Hg pressure acetone commences to vaporize and hence impregnation begins. This is the suggested pressure level to be reached after the first two hours of vacuum. With methylene chloride (MeCl), the two hour goal is to reach 18cm of pressure (MeCl's vapor pressure). Respectively, at RT their vapor pressures are 18cm and 35cm Hg (Pereira-Sampaio, 2006).

Solvent bubbles will usually be a bit smaller than the air bubbles. The goal for the end of day one: Decrease pressure from atmosphere (76cm) to 5cm Hg for either solvent (acetone or MeCl). As pressure is decreased below the solvent's vapor pressure, the acetone or MeCl vigorously boils out of the slices and lowers the temperature of the resin, which helps to control the exothermicity of the reaction-mixture (Fig. 6). Rate of impregnation (decreasing pressure) is monitored by formation of impregnation bubbles rather than relying solely on the vacuum level indicated by the manometer. Frequent regulation of the rate of evacuation is necessary to keep the impregnation boiling rate proper and hence keeps the temperature regulated between 0°C to 10°C which is the best range. Excessive bubble formation is an indicator of impregnating too fast. Resin level must be observed and more reaction-mixture added when necessary to keep the slices submerged.

Because of the variation of tissue volume and variation of impregnation-mixture volume used, it is not feasible to prepare a standardized vacuum curve for forced impregnation. However, it is beneficial to record vacuum adjustments and corresponding vacuum levels as well as the resin temperature to aid future projects and as a safety check on the current load if the impregnation cycle is interrupted. If impregnation conditions are kept constant, these data may facilitate the control of further impregnation runs.

By the end of the first day, the pressure should be reduced to 5cm Hg and during the second day, the pressure is decreased continuously down to 2mm Hg (at least 5mm Hg). When bubbling decreases considerably and big bubbles appear on the resin-mix surface and splash, impregnation is complete. Note: Bubbling may not completely stop. The best indicator that impregnation is complete is an increase of reaction-mixture temperature. Initially, impregnation temperature will decrease continuously, but at some point it will begin to rise. Standard impregnation takes 36-48 hours at +5°C. The elasticizer (AE10) in the impregnation-mixture increases the pot-life of the resin-mix. Impregnation at room temperature usually will be finished in 32 hours.

After impregnation is completed, the chamber is returned to atmospheric pressure and the glass port opened. The box containing the slices is removed and the foil/liner is discarded.

Curing

After impregnation is completed, the bundle containing the slices, grids and screen is lifted from the E12 impregnation-mixture and the excessive E12-mix drained (Fig. 7). There are two methods to cure E12 slices: The flat chamber method (using glass plates) and the sandwich method (using heavy foil sheets) both use the following *casting-mixture*: E12 - 95pbw, AE30 - 5pbw, E1 - 26pbw (AE10 is not used in this mixture to avoid soft flexible slices).

Casting slices in flat chambers: Flat chambers are assembled from 3mm thick safety glass plates, appropriate diameter gasket (silicone tubing or gasket or PVC gasket) and fold-back clamps (Fig. 8). To make a leak proof 35 x 45cm flat chamber, 10 fold-back clamps (3 on each side, 4 at the bottom and evenly spaced) are needed for silicone tubing. If other gasket material is used, more clamps are necessary for a good seal: 20 clamps for silicone gasket, or 32 clamps for PVC-

gasket. The length of the gasket for this size flat chamber is 150cm.

The appropriate size glass plate is placed on an assembly stand. A small pool of E12-mix is poured onto the glass plate to prevent the section sticking directly to the glass. The impregnated slice is placed on the pool of resin-mix on the glass plate. Next the silicone tubing is placed on the glass plate, 2cm from and parallel to the bottom edge of the glass. A spacer is placed in the middle of the top edge of the plate to support the top edge of the top glass which is placed into position on top of the tubing and spacer. The silicone tubing is held in position by four clamps, spaced equidistantly, starting 2cm away from each bottom corner. Next, the silicone tubing is turned upward, parallel to each side and clamps are positioned over the gasket, one each, near the top and bottom of each side and one in the middle. Consequently, all 10 fold-back clamps are placed on three sides of the flat chamber.

The flat chamber, containing the impregnated slice, is placed vertically in a supporting box so that its upper edge protrudes above the box. A flat funnel is used to fill the chamber with E12 casting-mix to about 8cm below the top (if placed properly, the bottom edge of each top side clamp can be used as a fill indicator. The pool of E12 resin-mix added earlier will facilitate positioning the slice allowing resin-mixture to flow on both sides of the section as the chamber is filled resulting in equal distribution of resin on each side of the section. After filling, flat chambers are placed for one hour in a vacuum chamber to remove small air bubbles present in the resin. Pressure is reduced to 2mm Hg and maintained for one hour. After returning to atmosphere, any remaining bubbles are removed manually using a 1mm stainless steel hooked wire of appropriate length. After bubble removal, the specimen is aligned in the flat chamber. The chamber is placed horizontally at an inclination of 15°C for at least 24 hours. During this period, the resin becomes more viscous preventing movement of slice. Finally, the flat chambers are placed upright in a 45°C oven for four days. After removal from the oven and allowed to cool down to room temperature, the glass plates are carefully removed. The epoxy slices are wrapped in foil and the sheets are cut to the desired size.

Casting of slices using the sandwich method: This method uses heavy foil (plastic) sheets. The main difference from the flat chambers is that this method is faster. The method uses both foil sheets and glass plates. Safety glass is not required for this procedure. Window or safety glass is placed on an assembly stand and a foil sheet, which must overlap the glass plate by 2cm on all sides, is placed on the glass. Fresh and deaerated casting-mixture is poured sparingly onto the polyester foil (Fig. 9). Impregnated slices are placed on the pool of casting-mixture on the foil (Fig. 10) and casting-mixture is spooned on top of the slices. The slices are gently covered with another foil (Fig. 11) and then a spatula is used to place pressure on the foil to squeeze and remove the air bubbles from the top of the slice (Fig. 12). The foil sandwich (top and bottom foil and slice) is turned over and the foil is squeezed with the spatula to remove air on this side of the slice. If there are more slices to be cured, the process starts again with a foil and resin-mix spooned onto the foil. A slice is placed on the resin and a top foil put into place. Air is squeezed from both sides of the sandwich. This foil sandwich is placed on top of the previous foil sandwich, etc. Three foil sandwiches are placed together; then a glass plate is introduced on top of the three foil sandwiches. If more slices are ready to cure, the process starts over again on the top glass: **glass** / foil / slice / foil / / foil / slice / foil / / foil / slice / foil / **glass** / foil / slice / foil / / foil etc. (Fig. 13).

Day 0	Freeze specimen -75°C.
Day 1	Slice and clean sawdust from slices.
Day 2	Immerse specimens in #1 -25°C acetone bath (>90%).
Day 5	Immerse in #2 bath, -25°C acetone (100%). Check and record purity of bath #1.
Day 8	Immerse in #3 bath, -25°C acetone (100%) Check and record purity of bath #2.
Day 11	Degrease slices in Acetone or MeCl @Room temperature (RT).
Day 18	Impregnate in E12 resin-mix (+5°C or RT).
Day 20 or 27	Cast or sandwich slices. Lay cast 15° from horizontal at RT.
Day 21 or 28	Cure upright in + 45°C oven.
Day 25 or 33	Open flat chamber or the sandwich, cover slice with foil, saw and sand.

Table 1. General protocol for epoxy slices (2-3mm).

When the sandwich stack is finished, a glass plate is placed on the top. A tower of foil/slice/foil sandwiches is formed with glass on the top and bottom and after every 3rd sandwich, which reminds one of a sandwich. If slices are not of uniform thickness, a glass plate is only used on the bottom and the top of the stack after all of the foil sandwiches are made. This will help assure more uniformity of slices. In order to keep the foil plates pressed and to encourage excess E12 resin to

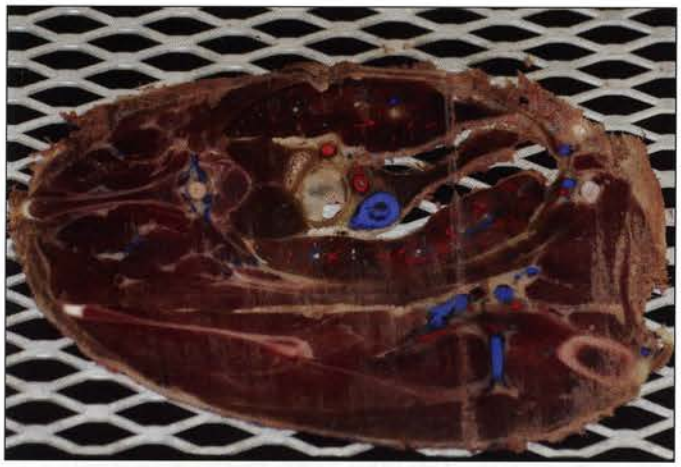

Figure 1. Fresh tissue slice with saw dust, lying on metal grid.

Figure 2. Accessories needed for stacking and bundling slices: Fly screen (f), grids - metal (m) and plastic (p), basket and reservoir.

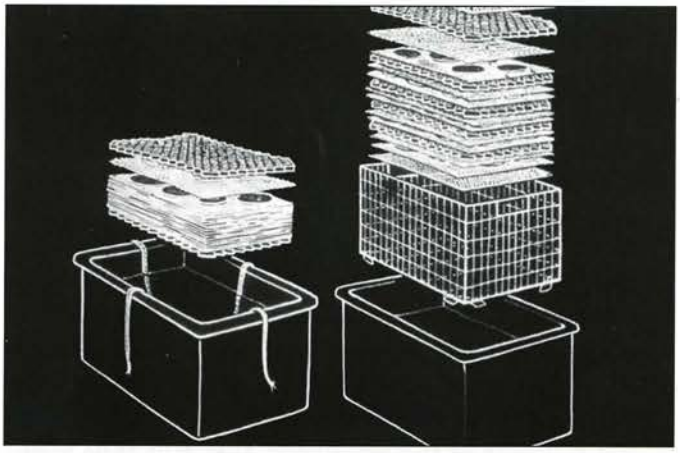

Figure 3. Slices ready to be bundled and tied with twine or placed in basket.

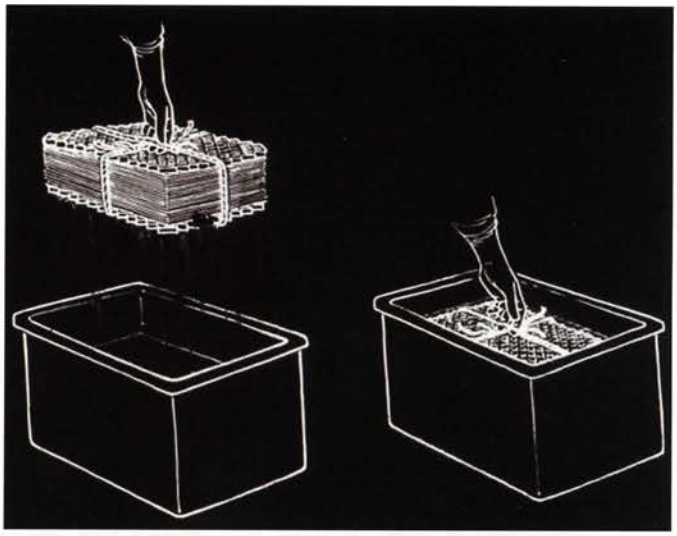

Figure 4. Changing bundled slices into new acetone bath.

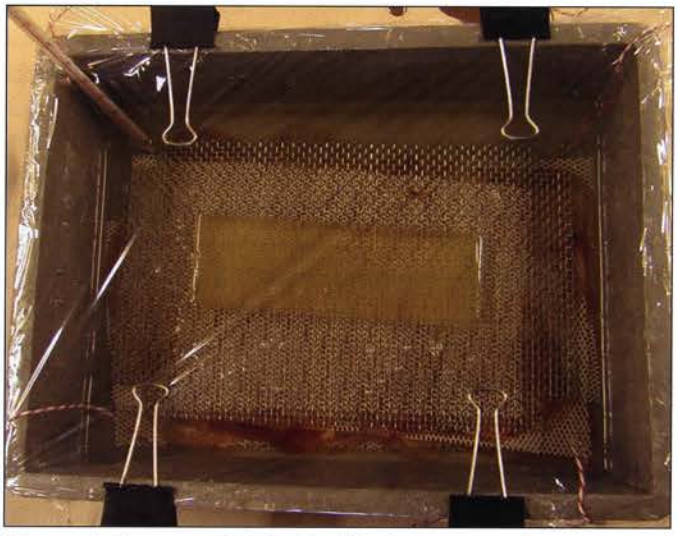

Figure 5. Impregnation bath with thermometer in corner and covered with foil.

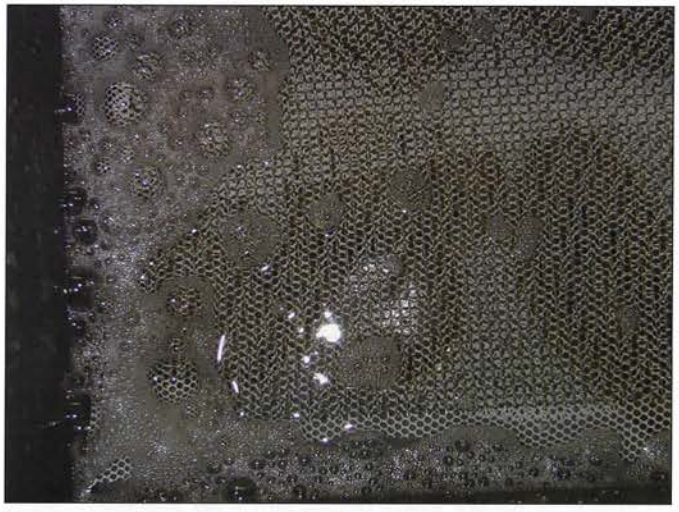

Figure 6. Impregnation bubbles being produced at an appropriate rate.

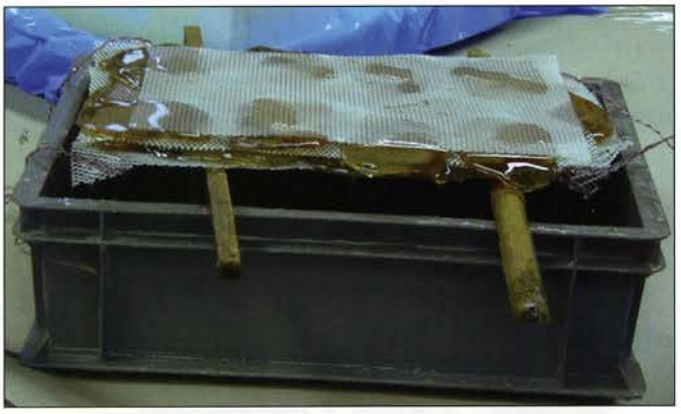

Figure 7. Impregnated slices draining of excess E12 resin-mix.

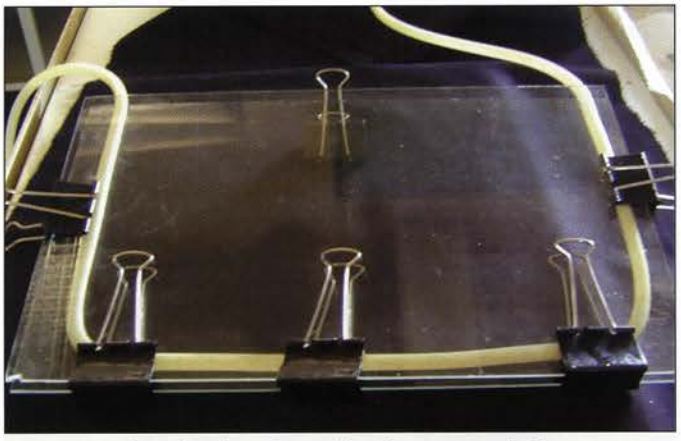

Figure 8. Flat-chamber for curing impregnated slices.

Figure 9. Sandwich curing technique: Casting-mixture poured onto heavy foil overlying the glass plate in preparation for impregnated slice.

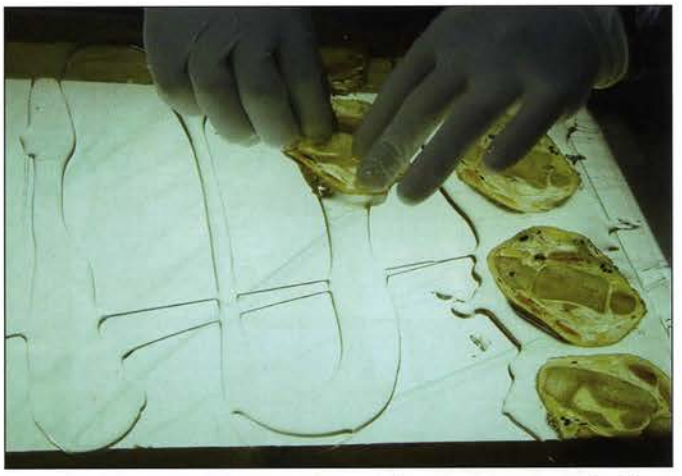

Figure 10. Sandwich curing technique: Impregnated slices placed on casting-mixture.

Figure 11. Sandwich curing technique: Covering slices with foil.

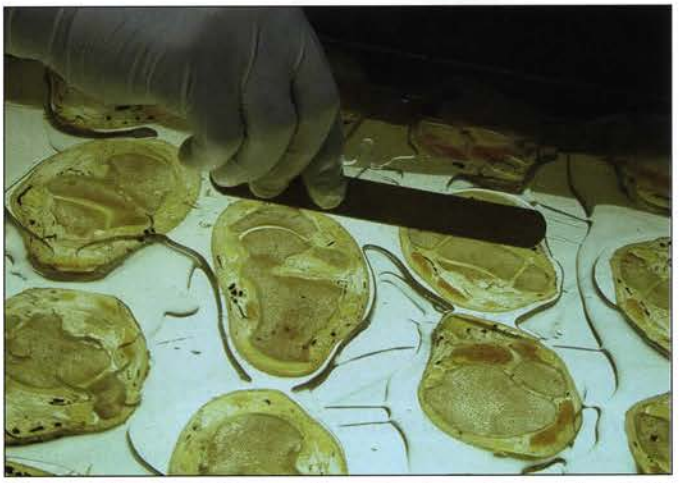

Figure 12. Sandwich curing technique: Removal of air bubbles using a spatula.

Figure 13. Sandwich curing technique: Glass is placed over foil.

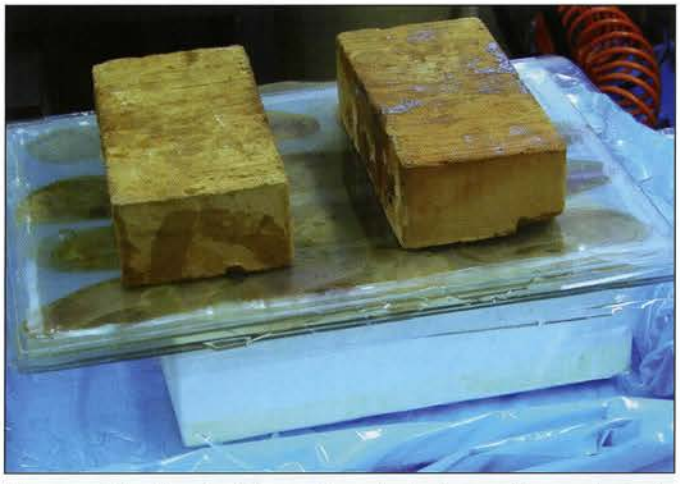

Figure 14. Sandwich curing technique: Top glass is weighted.

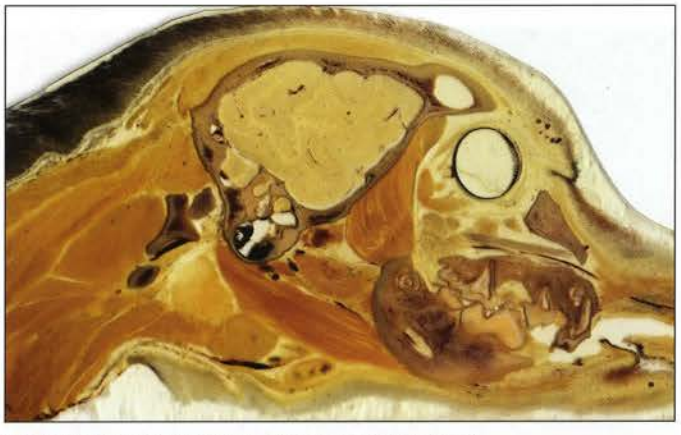

Figure 15. E12 sagittal slice of canine head.

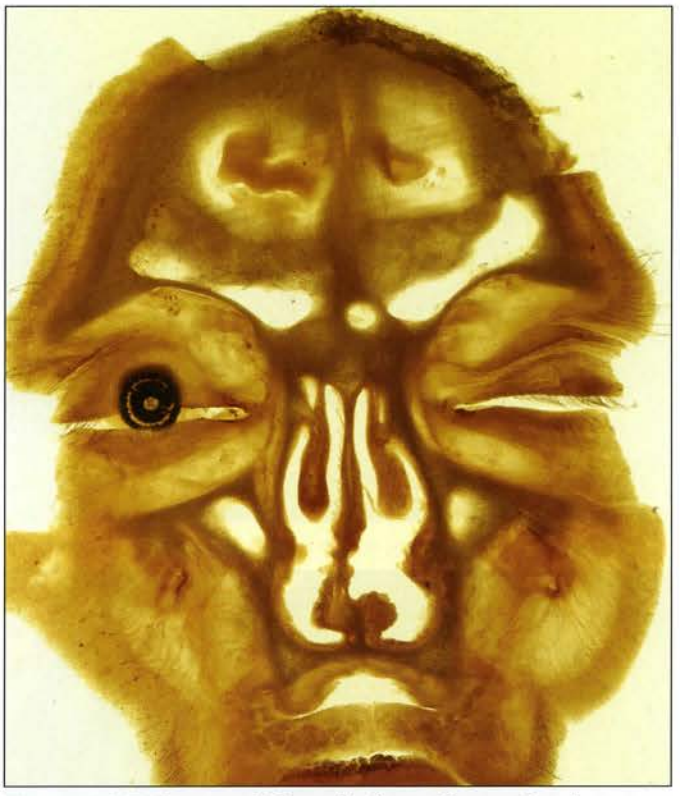

Figure 16. E12 coronal (frontal) slice of human head.

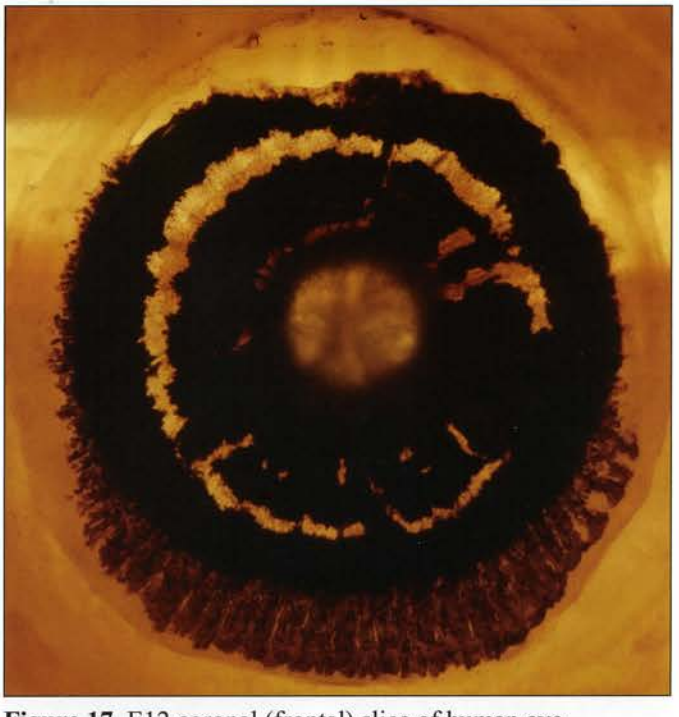

Figure 17. E12 coronal (frontal) slice of human eye.

ooze out, a weight is placed upon the top glass plate (Fig. 14). This sandwich block remains at room temperature for 1 day and then is placed in a +45°C oven for four days. After the sandwich has been removed from the oven and cools to room temperature, the foil sheets are carefully removed and the resin plates containing their specimen slice are cut as desired.

Results

The transparency and color of the plastinated E12 slices are of the highest quality. The finished E12 sections are durable, semi-transparent and correlate readily with radiographs when placed on light boxes and above all else offer more detail than is possible with any other plastination method or gross dissection (Figs. 15-17). Successful results with the E12 technique are dependent upon carrying out the specific parameters for section thickness, lipid extraction and impregnation technique.

Discussion

The main advantage of the E12 sheet plastination method is that it preserves the topography and integrity of a target region in a complete and uninterrupted state with all interconnected structures on a given plane preserved. The slices can be stored at room temperature and used later on for further investigations. Because of this, morphologic measurements can be performed easily. The data thus obtained are comparable to that of high quality magnetic resonance (MR) images (Beyersdorff et al., 2001; Steinke, 2001; Thomas et al., 2003). The one slight disadvantage is: epoxy slices tend to yellow in color over a period of years.

The removal of lipid content from tissue slices prior to impregnation is an essential step for the success of the E12 technique. Acetone, as well as being the recommended medium for dehydration by freeze substitution, serves as an adequate degreasing solvent when used at room temperature. Although not mandatory, experience demonstrates that the highest optical quality for El2 sections was achieved from tissue sections that had been degreased first in acetone at room temperature and then transferred to methylene chloride for more lipid extraction.

For research these slices allow topographical study of all body structures in an uncollapsed and non-dislocated state. Also, specimens are useful in advanced sectional topography programs and resident training in CT and MR. Computerized reconstruction of anatomical structures is becoming useful for developing anatomical and research teaching modules and animations. E12 slices will likely play a significant role in these areas.

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Product distribution:

Biodur Products, Dr Andrea Whalley, Rathausstrasse 11, 69126 Heidelberg, Germany. www.biodur.de, Ph: (49) 6221 3311-11, Fax: (49) 6221 3311-12.

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Epoxy Plastination of Biological Tissue: E12 Ultra-thin Technique

M-C. SORA

[1]*Plastination Laboratory, Centre for Anatomy and Cell Biology, Plastination and Topographic Anatomy, The Medical University of Vienna, Währingerstr. 13/ 3, A-1090 Wien, Austria.*

[*]*Correspondence to: Telephone:* 43-1-4277-611-50; *Fax:* 43-1-4277-611-70; *E-mail:* mircea-constantin.sora@meduniwien.ac.at

Abstract: The E12 method of plastination classically is used to create thin 2-5mm transparent slices. If thinner slices (0.5-1.0mm) are desired, it is necessary to use the ultra-thin slice plastination method. By using this method, the specimen is first plastinated as an epoxy block and then cut into thin slices using a diamond saw. The impregnation temperature and the percent of accelerator (E600) used in the resin-mix are the key elements necessary to obtain proper impregnation of the desired tissue block and contrary to all other plastination methods, high temperature (30 to 60°C) is desired. This paper describes the use of high temperature in the epoxy process to produce ultra-thin (<1mm) epoxy plastinated slices. Only by using high temperature is the resin thin enough to penetrate into the middle of a large specimen.

Key Words: **ultra-thin plastinated slices; E12/E6 impregnation; high temperature**

Introduction

Temperature is usually a crucial factor in plastination. Usually, during dehydration, degreasing or epoxy (E12) impregnation of slices, the temperature does not exceed room temperature values. High temperature, up to 45°C, is only used during curing. "Why is high temperature not used?" High temperature is not used during dehydration and degreasing because high temperature increases shrinkage and during impregnation the E12/E1 impregnation mixture becomes very viscous within hours, preventing complete impregnation. The exception is E12 block impregnation which uses E6 as a hardener and utilizes high temperature (60°C) for the end of impregnation as well as curing.

Classically, thin slices (2-5mm) of the desired specimen are prepared by epoxy plastination. Cold dehydration, degreasing, impregnation and finally curing similar to other types of plastination are used (von Hagens, 1986; Weber and Henry, 1993; Cook and Ali, 1997; Fasel et al., 1988; An and Zhang, 1999; Sora et al., 2002; Sora et al., 2004). However, ultra-thin slice plastination produces epoxy slices with a thickness of 1mm or less (Fritsch and Hegemann, 1991; Seibold et al., 1991; Sittel, 1996; Johnson et al., 2000; Windisch and Weiglein, 2001). A different approach is needed to produce the ultra-thin slices which can not be made by conventional sawing of the frozen specimen on a bandsaw. The entire desired specimen block is dehydrated and degreased as a unit and hence impregnated and cured as a block. Finally the cured specimen is sliced into ultra-thin E12 slices using a saw with a special diamond blade. This technique presents the utility of high temperature in processing and creating cured E12 blocks for sawing ultra-thin plastinated slices. A major problem of impregnating bigger tissue blocks is to get the E12 resin-mix into the middle of the block. At room temperature, the E12 resin is liquid but gets thicker when temperature is decreased.

If temperature is increased, the E12 resin alone becomes very fluid. However, increasing the temperature of the E12/E1 reaction-mixture causes the resin-mixture to become too viscous to impregnate tissue. If a different hardener (E6) is used, the marked increase in viscosity is delayed for 4-5 days. Biodur E6 is an anhydride-based hardener of low viscosity which causes no immediate thickening of the resin reaction-mixture. This permits impregnation of the tissue block at 30°C to 60°C (von Hagens, 1986).

Materials and methods

The standard steps of ultra-thin E12 slice plastination are specimen selection and preparation, cold dehydration, degreasing, impregnation and curing of the tissue block. From this cured block, 1mm thick or less slices are made after impregnation and curing (Fritsch and Hegemann, 1991; Seibold et al., 1991; Sittel, 1996; Johnson et al., 2000; Windisch and Weiglein, 2001).

Specimen preparation

The desired specimen is selected and may or may not be fixed with formalin. Fixation is not necessary for production of semi-transparent slices using E12 ultra-thin plastination. In general it may be desirable to fix tissue to prevent any potential biohazard risk which may accompany handling and sawing of human or animal tissue. The disadvantage associated with tissue fixation is the marked loss of tissue color. A large specimen block should be cut into the desired sized blocks for impregnation, each with a maximum size of 15cm x 10cm x 10cm, as well as positioned in proper anatomical position prior to freezing or fixation.

Dehydration and degreasing

Freeze substitution is the standard dehydration procedure for plastination. Shrinkage is minimized when cold acetone is used. The tissue block is placed into a -25°C freezer for two days and then submerged in cold (-25°C) technical quality 100% acetone for dehydration. The ideal acetone to specimen ratio should be 10:1. Length of dehydration will be proportionate to the size of the tissue block. For assurance of thorough dehydration, acetone baths should be monitored and not changed until acetone purity is stable. Check the first acetone bath for purity at two and three weeks. If the acetone percent remains constant, place the specimen into the second cold acetone bath; or if acetone purity has decreased <2%, monitor the purity after one more week. When acetone purity is stable, change the specimen into the second acetone bath. Check acetone purity of the second bath at two and three weeks. If acetone purity remains constant, place the specimen into the third cold acetone bath. Check this third bath for purity at two and three weeks. If the acetone purity is >98%, dehydration is complete. Bring the acetone and tissue block to room temperature. Replace the warm acetone with methylene chloride (MeCl) for degreasing. MeCl is a stronger degreaser than acetone. However, it is a hazardous substance and must be handled and kept in a ventilated hood. Since there is no method to measure the fat concentration of the used MeCl, the color of the bath is monitored for change to yellow. When the color becomes an intense yellow, it is changed for new MeCl. Degreasing should continue for at least two weeks to assure a fat-free block for impregnation. Larger blocks and or blocks with more fat should degrease even longer up to four weeks. To assure adequate degreasing, it is necessary to visually inspect the quality of the specimen. Degreasing is complete when the fat is transparent.

Forced Impregnation

Forced impregnation is the key to plastination. During impregnation, the solvent is extracted from the cellular and interstitial space of the specimen and replaced with the resin impregnation-mix [E12 (resin)/ E6 (hardener)/ E600 (accelerator)] (von Hagens, 1986). The epoxy resin and hardeners are mixed as follows to prepare the E12 *impregnation-mixture*: **E12 - 100pbw, E6 - 50pbw, E600 - 0.2pbw**. The dehydrated/degreased specimen is removed from the methylene chloride bath and submerged in the E12 impregnation-mixture [E12 (resin)/ E6 (hardener)/ E600 (accelerator) (100/ 50/ 0.2)]. The reservoir of resin-mix and specimen are placed into the vacuum oven set at +30°C, such as a Heraeus VT 6130 M from: Heraeus Instruments, Kendro Laboratory Products GmbH. Vacuum is not applied until the next morning in order to allow the E12 resin-mix to equilibrate and commence penetration of the specimen. The next day vacuum is applied and stabilized at 40cm Hg pressure at +30°C. Over five days, pressure is reduced 8cm Hg daily, until 2mm Hg is reached. The first four days temperature remains at 30°C. By the fourth day, the resin-mix is becoming more viscous. On the fifth (last) day, temperature is increased to 60°C to make the resin-mix as fluid as possible thus able to penetrate the depths of the specimen (Table 1). It is important to monitor viscosity closely at this time as the E12/E6/E600 mixture becomes thinner at first. However, after several hours, bubbles begin to rise slower and splash intensely as the resin-mix again becomes more viscous. This indicates the onset of polymerization and it is time to stop impregnation.

Curing

When impregnation is completed, the tissue block is removed from the vacuum chamber. A mold is constructed of Styrofoam and lined with polyethylene

foil and the tissue block inserted. Fresh resin reaction-mixture **[E12/ E6/ E600 (100/ 50/ 0.2)]** is used to fill the mold and surround the specimen. The mold containing the impregnated specimen and resin-mix is placed in a 65°C oven for four days to harden the resin-mix. The tissue/resin block is cooled to room temperature and the mold removed (Figs. 1, 2).

Slicing

A contact point diamond blade saw, Exact 310 CP (Exact Apparatebau GmbH, Norderstedt, Germany) or equivalent is used for cutting the block (Fig. 3). The hardened E12 block is cut into 1mm or slightly less slices (Figs. 4, 5). Between each slice tissue, the width of the saw blade (0.4 mm), is lost.

The general protocol for Ultra-thin Epoxy (<1mm) Slices from the frozen (-25°C) specimen is listed in Table 1.

Day 1	Immerse in #1 bath -25°C acetone >90%.
Day 14	Check and record purity of acetone bath #1, Immerse in #2 bath, -25°C acetone (100%).
Day 28	Check and record purity of acetone bath #2. Immerse in #3 bath, -25°C acetone (100%).
Day 42	Degrease in MeCl @ Room temperature (Minimum 2 weeks).
Day 56	Immerse in E12 resin-mix (+30°C).
Day 57	Impregnate in E12 resin-mix (+30°C).
Day 61	Impregnate in E12 resin-mix & increase temperature to +60°C.
Day 62	Cure specimen +65°C oven.
Day 66	Remove block and slice when convenient

Table 1. Ultra-thin epoxy slice (<1mm) protocol.

Results

A plastinated specimen is produced and incorporated in an E12/tissue block. This block is hard and semi-transparent. Ultra-thin, <1 mm slices are produced from this block (Figs. 4, 5). These ultra-thin slices have excellent optical qualities and are hard, as well as transparent. Ultra-thin E12 slices provide excellent anatomic detail down to the microscopic level (Fig. 6).

Discussion

For thorough impregnation, the resin/hardener-mixture must be fluid enough to penetrate the specimen. Also the processing time must be of sufficient length to penetrate to the core of the tissue block. Epoxy resin viscosity has an inverse relationship with temperature: At low temperature resin viscosity is high, while at high temperature resin viscosity is low. With impregnation at low temperature, the epoxy resin becomes viscous. Low temperature prolongs processing time. However, since the E12-mixture is reactive, it will become too viscous in two days. Hence this is the rational for the standard E12 method (E12 resin/ E1 hardener-mix). Because of the rapidly increasing viscosity of the reaction-mixture, only thin slices (2-5mm) may be impregnated.

The ultra-thin epoxy technique produces slices by sawing <1 mm (ultra-thin) plastinated slices from an E12 impregnated specimen block. To impregnate a large tissue block, a low viscosity reaction-mixture is used and the impregnation time must be increased. The E6 hardener fulfills this need and is augmented by an increase in impregnation temperature. E12/E6 processing time is also controlled by the quantity of accelerator (E600) added to the resin-mix. Both high temperature and an increased quantity of E600 speed up polymerization time and hence processing time is decreased. By adding less E600, impregnation time can be extended a few days. Submerging the tissue block in the reaction-mixture overnight allows the E12/ E6/ E600 resin-mix to equilibrate and begin penetration into the specimen. The methylene chloride which enters the resin-mix lowers the viscosity of the reaction-mixture. The E12/ E6/ E600 mix is quite viscous at room temperature. However, by using a temperature of +30°C, decreases viscosity significantly and viscosity of the reaction-mix remains low for four days. Lower viscosity aids both the extraction of the solvent and influx of the resin-mix. The vacuum drying oven allows simultaneous adjustment of vacuum and temperature. On the fifth (last) day, to counter the increasing viscosity of the resin reaction-mixture, the temperature is increased to 60°C. Viscosity must be monitored closely as it becomes thinner at first. But, after several hours, the resin-mix becomes more viscous and impregnation must stop.

Using the E6/E600 hardener delays polymerization of E12 and allows impregnation of large tissue blocks. Curing time and firmness depend on the quantity of accelerator E600. An accelerator (E600) is needed for the resin hardener-mix to polymerize. However only a small amount of accelerator is used 0.2%. Larger amounts of the accelerator in the E12/ E6 mix will cause curing of the block too soon. If no E600 is used, polymerization of the block will not occur, even if temperatures of 60°C are maintained for several months (von Hagens, 1986). The plastination folder contains a table listing the reaction and hardening time for a variety of E12/E6/E600 mixtures.

For histological studies (Fig. 6) and accurate 3D reconstruction (Fig. 7) of plastinated slices, ultra-thin slices (<1mm) are essential (Sha, 2001; Qiuet, 2003). A diamond saw is essential to cut ultra-thin slices from the

Figure 1: E12 impregnated and cured pelvic block.

Figure 3: Diamond saw slicing E12 pelvic tissue block.

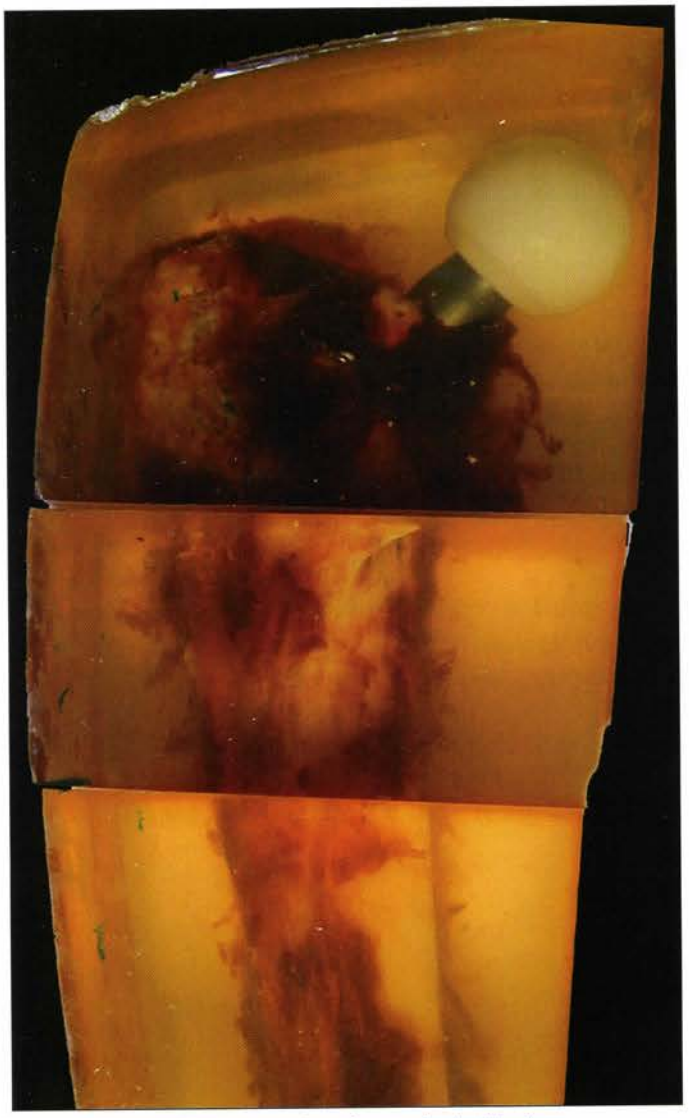

Figure 2: E12 impregnated endoprosthetic block.

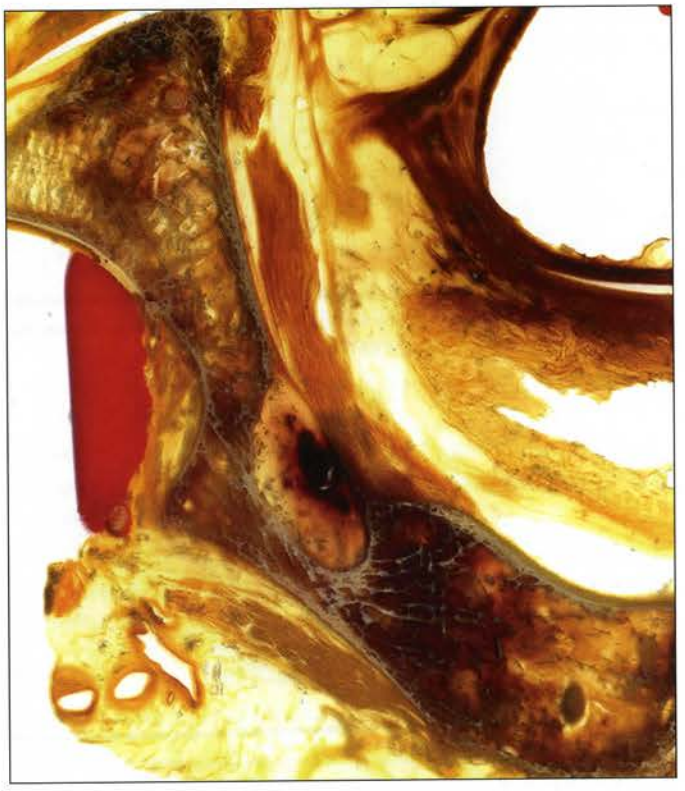

Figure 4: Ultra-thin slice of the pelvis block from figure 1.

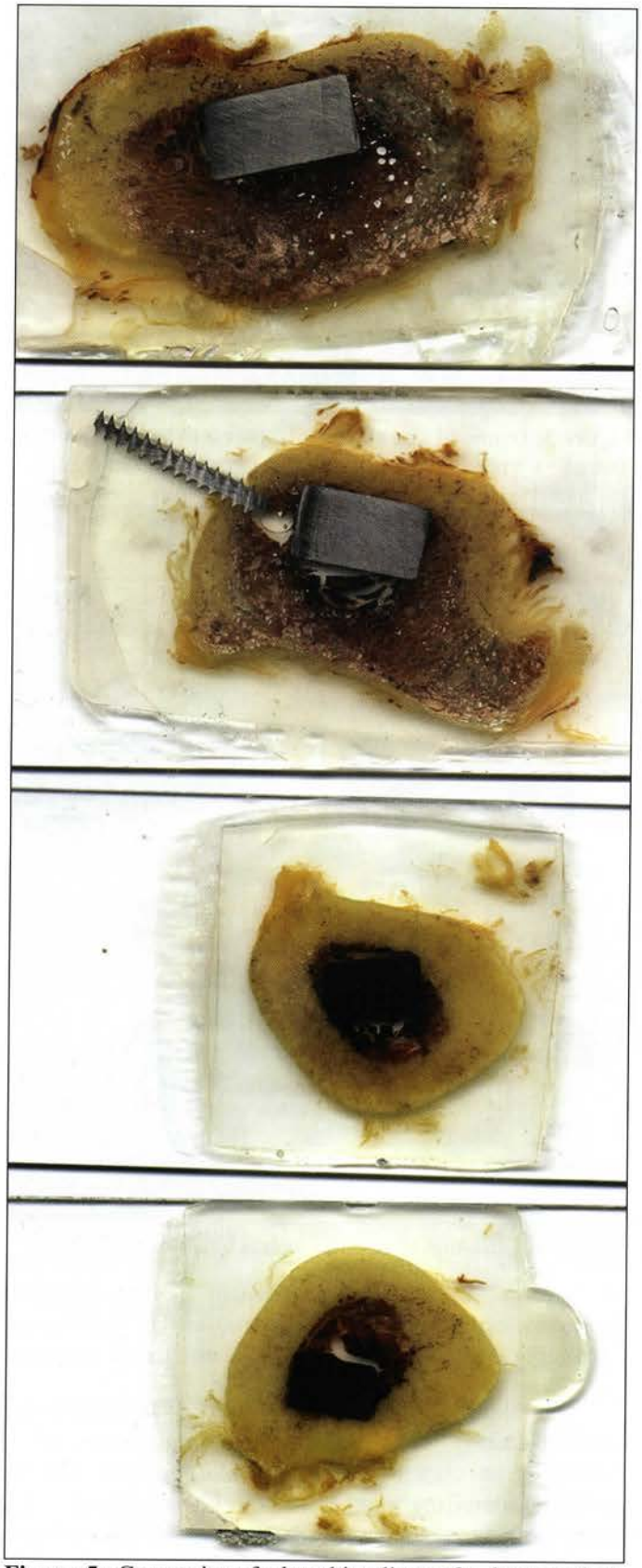

Figure 5: Composite of ultra-thin slices of a bone and its prosthesis.

Figure 6: Photomicrograph of bone (40X) from figure 5.

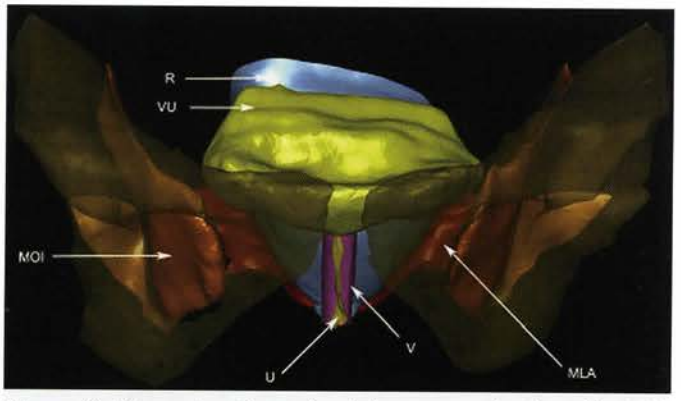

Figure 7: Reconstruction of pelvic organs via ultra-thin E12 slices. MOI - Musculus obturator internus, R - Rectum, V - Vagina, VU - Vesica urinaris, MLA - Musculus levator ani.

E12 impregnated tissue block. Histological examination can be performed up to a magnification of 40X. Because of the thickness (>300 microns) of the specimen, higher magnification is not possible (Sora, 2002). The normal methods for staining histological specimens may be used (Gruber, 2001). An important advantage of ultra-thin epoxy slices is that structures remain intact and decalcification of bone is not necessary. Since topography of a region may be studied in a non-collapsed and non-dislocated state, morphological measurements can be performed accurately and easily.

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Product distribution:

Biodur Products, Drs. Gunther von Hagens and Angelina Whalley, Rathausstrasse 11, 69126 Heidelberg, Germany. www.biodur.de, Ph: (49) 6221 3311-11, Fax: (49) 6221 3311-12.

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Epoxy Plastination of Biological Tissue: VisDocta EP73 Technique

T. SHAHAR[1], C. PACE[1] and R.W. HENRY[2]

[1]*VisDocta Research, Via Panoramica 2B, Tignali (BS) 25080, ITALY, Europe.*
[2]*Department of Comparative Medicine, College of Veterinary Medicine, University of Tennessee, Knoxville, TN, USA.*

Correspondence to: Telephone: 39 (3) 65 761 000` *Fax:* 39 (3) 65 761 900; *E-mail:* visdocta@tin.it

Abstract: Since its development over 20 years ago, sheet plastination has become one of the premier methods for preservation of perishable biological human and animal tissue slices. With the advent of modern imaging techniques, the need for interpretative aids is evident. Production of slices using the epoxy technique, has served this purpose well. Because of the importance of this technique which has good optical, preservation and presentation qualities and the success of epoxy in this role, a similar generic product was developed by VisDocta in Italy. This generic resin yields transparent slices which exhibit detailed structures in their normal anatomical position similar to the Biodur™ E12 method. In addition, yellowing of the cured resin is not a problem

Key words: **plastination; epoxy; transparent body slices; VisDocta; VS-EP73; VS-EP70H**

Introduction

The purpose of epoxy sheet plastination is to preserve and present 2-4mm slices of tissue for examination/ study by replacing tissue fluid and some lipid with a curable resin. This protocol describes plastination of sliced body parts via a generic epoxy method. Body slices (2-4mm thick) are produced using a flat-glass chamber. The basic tenants of plastination are followed. All water and most all of the lipid are removed from the tissue and curable epoxy resin-mix replaces them (Weber and Henry, 1993).

Also, as with other methods of plastination, this epoxy technique may be modified and/or some steps may be omitted. However, if this standard protocol of this epoxy technique is followed, the best results are obtainable.

The importance of understanding sectional anatomy of man and animals is underscored by today's modern diagnostic imaging techniques (computed tomography, magnetic resonance imaging, and ultrasound). The epoxy plastination method provides durable semi-transparent sectional preparations of excellent visual acuity.

Chemicals used in this "generic" epoxy-plastination process are similar to the ones used in the Biodur™ E12 sheet plastination technique:

- Acetone
- Methylene chloride (MeCl), for defatting
- Epoxy resin
- Hardener, to link the epoxy molecules

The general steps of epoxy plastination are described earlier in this volume. This manuscript highlights the differences with respect to this generic process which occur with impregnation and curing of the plastinated specimens and utilizes similar specimen preparation, dehydration and defatting techniques as any standard plastination technique.

Materials and methods

The basic steps of plastination are utilized for each process:

Specimen preparation, dehydration, defatting

Specimen preparation, dehydration, defatting (degreasing) are the same for all plastination methods including the epoxy technique. As well, portions of the forced impregnation and casting/curing steps will refer back to the standard "Biodur™ E12 plastination technique" for more detailed information.

Forced Impregnation

Replacing the volatile solvent (acetone/MeCl) in a biological specimen with a curable resin. For this to occur, the chemicals must meet similar criteria as the chemicals used in the Biodur™ E12 plastination technique.

Impregnation equipment: Similar to the Biodur™ E12 Epoxy Technique - Vacuum pump, Vacuum chamber, Vacuum gauge, Bennert mercury or digital manometer, Needle valves for regulation of pressure, Specimen basket and grids, and cold room.

VisDocta products for **epoxy plastination:**

VS-EP73: Epoxy resin
VS-EP70H: Hardener

Preparing impregnation-mixture:

Forced impregnation is the unique process in plastination. Solvent is vaporized from the specimen by decreasing pressure and is simultaneously replaced with the resin-mix. To prepare the EP73 *impregnation-mixture*, the resin and hardener are mixed as follows: **EP73 - 100 pbw** with **EP70/H - 14 pbw**. Mix the correct volume of reaction-mixture to adequately cover the volume of slices for impregnation. The reaction-mixture is placed in the impregnation vat at room temperature. The bundle of dehydrated and degreased, stacked slices, grids and screens are transferred from the degreasing bath into the room temperature impregnation bath. Pre-plan the size of the specimen impregnation reservoir to adequately hold the stack of slices. The plan should include minimizing the amount of space around the slices in the reservoir. This will keep the volume of EP73 resin reaction-mixture needed to fill the reservoir at a minimum.

Make sure that the slices are covered with an adequate volume of resin-mix. As with most epoxy reaction-mixtures, EP73 has a short pot life (8 days at 0°C) which cannot be extended. Careful planning will reduce the amount of resin-mix needed as well as reduce the cost of the slices.

Impregnation regimen: *Similar to the Biodur™ E12 epoxy proceedure.

Adjusting the vacuum: (Speed of lowering the pressure) The reservoir containing the slices and the EP73-EP70/H impregnation-mixture is placed in the vacuum chamber in a cold room environment; 2°C is preferred with a maximum of 5°C. The lower temperature prolongs the pot life and allows slower extraction of the solvent. Actual impregnation starts around 20cm/8in Hg when the methylene chloride degreasing solvent reaches its vapor pressure or 8cm/3in Hg for acetone.

Vacuum is applied and pressure is lowered moderately with frequent smaller closures of the air-intake valve (lowering of pressure) to initially keep a continual evacuation of the trapped air from the chamber. Smaller more frequent adjustments help avoid rapid boiling of the exiting air (degassing/deaerating). Around 20cm Hg pressure solvent extraction begins and this pressure is usually reached after eight hours of slowly decreasing pressure.

Once solvent begins to vaporize less frequent closure of the intake valve is necessary to keep the solvent boiling. However, valve closure should be minimal, just enough to keep the solvent boiling slowly. A moderate to slow impregnation speed is desirable over a seven day period. Boiling speed (solvent extraction) will vary with the quantity of solvent in the system and with temperature (Fig. 1). Both increased solvent quantity and increase in temperature will increase boiling rate. Excessively rapid boiling may result in resin over flow and the top slices losing the resin that covered them. If this occurs, adequate resin-mix must be added to cover the slices.

Pressure is continuously reduced slowly down to 1-2cm Hg over a seven day period with a maximum of eight days. Impregnation is complete when bubble activity stops.

Casting and curing (hardening)

The resin in the slices must be hardened. Casting and curing is similar to the Biodur™ E12 proceedure.

The impregnated bundle of slices with the grids and screen are lifted from the EP73 impregnation-mixture and drained. Either of the two standard casting methods may be used to cast and cure the impregnated slices: Flat chamber method (using paired glass plates) or the Sandwich method (stacking the slices between foil). Slices, placed in the flat chambers, use chambers constructed from an appropriate size of glass, fold back clamps and gasket. PVC gasket which will react with MeCl is not recommended. For the flat chamber method: Teflon or polyethylene gasket material are preferred since they have better physical and chemical resistant properties. Slices cured via the sandwich method use a glass plate at each end of the stack which is covered with heavy Mylar sheets. Mylar sheets then are used between each slice. Mylar sheets are more

chemical resistant. Impregnated slices are placed between Mylar sheets similar to the Biodur™ E12 Technique. The following *casting-mixture*: **EP73 - 100 pbw** with **EP70H - 14 pbw** is used.

Deaerating slices: *Variation from the Biodur™ E 12 proceedure. After filling, the flat chambers are placed in a vacuum chamber to remove small air bubbles (degas/ deaerate) in the resin. Pressure is reduced gradually to 49cm/19in Hg (600mbr) over a one hour period. Then over the next eight to ten hours, pressure is lowered to 34cm/13in Hg (450mbr). Care must be taken not to decrease pressure too rapidly and boil the air out of the resin too fast.

Day 0	Freeze specimen -75°C.
Day 1	Slice and clean sawdust from slices.
Day 2	Immerse specimens in #1 -25°C acetone bath (>90%).
Day 5	Immerse in #2 bath, -25°C acetone (100%). Check and record purity of bath #1.
Day 8	Immerse in #3 bath, -25°C acetone (100%) Check and record purity of bath #2.
Day 11	Degrease slices in acetone or MeCl @ Room temperature (RT).
Day 18 MeCl or Day 25 Acet.	Impregnate in EP73 resin-mix (2°C to 5°C) for 7 days (8 days maximum).
Day 25 or 32	Cast or sandwich slices. Deaerate cast slices.
Day 26 or 33	Cure specimens at 25°C for 7 days.
Day 33 or 40	Open flat chamber or sandwichs and cover slice with foil (plastic wrap), saw and sand.
Day 33 or 40	Optional! Harden 5 days in +50°C oven.
Day 38 or 45	Open flat chamber or sandwichs and cover slice with foil (plastic wrap), saw and sand.

Table 1. General protocol for the VisDocta EP73 technique for epoxy slices (2-4mm).

Curing slices: *Variation from the Biodur™ E 12 proceedure.

To cure the cast slices, the deaerated flat chambers are placed upright and allowed to cure at room temperature provided the temperature is >+18°C for seven days. However, 25°C is best and the maximum temperature for curing. After curing, the epoxy slices may be tempered by placing in a +50°C oven for five days to increase the durability of the manufactured slices. After curing and/or tempering, the glass plates are carefully removed. The slices/sheets are covered with thin foil and are cut to the desired size. The general protocol for production of epoxy slices from frozen specimens using the VisDocta technique is listed in table 1.

Results

The finished EP73 slices are durable and semi-transparent and correlate with radiographs and images of other modern imaging techniques (Fig. 2) (Henry et al., 1997). As with the Biodur™ E12 technique, results are dependent upon specific parameters of section thickness and lipid extraction being adhered to. The transparency and color of the plastinated EP73 slices are of the highest quality. The EP73 slices do not yellow with age (Fig. 3). No oven is needed for curing; however an oven may be used for tempering. Hardening, yields better physical characteristics of the final slices: Better scratch resistance and will not soften if heated.

Discussion

Epoxy sheet plastination maintains the integrity and topography of a region as a unit in a given plane (Cook, 1997; Henry et al, 1997; Windisch and Weiglein, 2001; Zöggeler et al., 2002). The slices are storable at room temperature and may be conveniently stacked. The advantage of this newer generation epoxy resin is a decrease in the post-cure yellowing of the resin and hence a more transparent final product for a longer period of time. Yellowing to browning of the epoxy sheet has been a criticism of the E12 sheets (Latorre et al., 2002a; 2002b; Reed and Henry, 2002).

An oven is not necessary for curing. The optimal curing temperature is 25°C. However, ambient temperature must be at least 18°C with a maximum of 25°C. If curing temperature is lower than 18°C, polymerization will be too slow and the physical and chemical characteristics of the slice may be of a lower quality. However, oven curing/tempering may be used with the VisDocta products. The EP73 technique is comparable with the E12 technuique.

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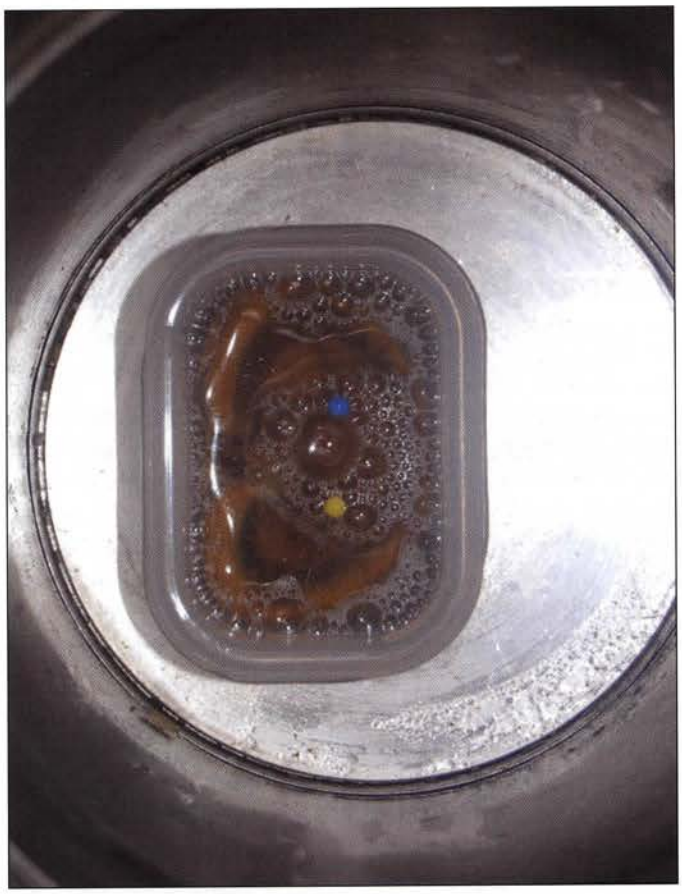

Figure 1. Approximate rate of bubble production for EP73 impregnation.

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Product distribution:

VisDocta Research Laboratories, Dr. Tsabari Shahar, via Panoramica 2B, 25080 BS Tignale, Italy. visdocta@tin.it, http://www.visdocta.com/, Ph: 39 (3) 65 761 000, Fax: 39 (3) 65 761 900.

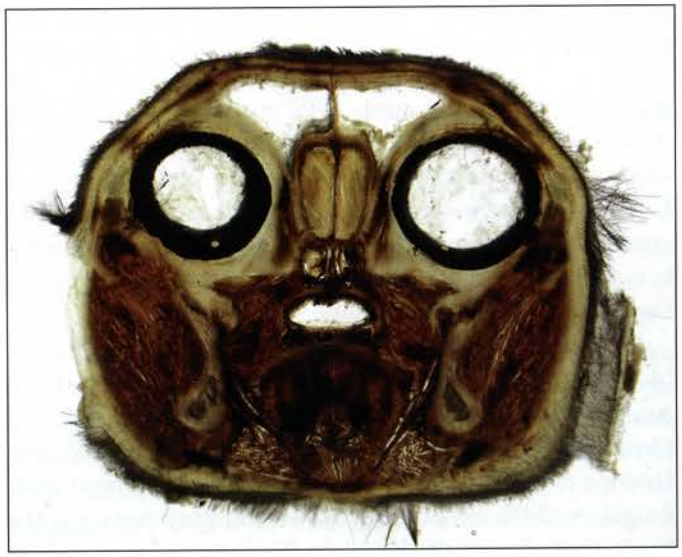

Figure 2. Impregnated, cast and cured transverse section of domestic feline head.

Figure 3. Comparison of cured block of early generation epoxy (left) and cured block of EP73 (right) six years post-curing.

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Polyester Plastination of Biological Tissue: P35 Technique

W. WEBER[1], A. WEIGLEIN[2*], R. LATORRE[3] and R.W. HENRY[4]

[1]*Cell biology-Anatomy, College of Veterinary Medicine, Iowa State University, Ames, IA.*
[2]*Anatomishces Institute, Medical Universitat Graz, Graz, Austria, Europe.*
[3]*Anatomia y Embryologia, Facultad de Veterinaria, Universidad de Murcia, Murcia, Españia, Europe.*
[4]*Department of Comparative Medicine, College of Veterinary Medicine, University of Tennessee, 2407 River Drive, Knoxville, TN, 37996-4543, USA.*

[*]*Correspondence to: Telephone:* (865) 974 - 5822; *E-mail:* andreas.weiglein@meduni-graz.at

Abstract: Plastination has become the gold standard for preservation of perishable biological tissue. Plastination is used in various anatomical, clinical and biological methodologies. The Biodur™ polyester technique produces 4-8mm translucent slices which display sectional anatomy within its normal anatomical relationships. These slices are superb aids to study and understand modern diagnostic images of computed tomography, magnetic resonance and ultra sound. Historically, brain tissue has been sliced and utilized for this technique.

Key words: **plastination; polyester method; body slices; polyester resin; P35**

Introduction

Polyester sheet plastination was developed for preservation of brain tissue for teaching and research. The slices are prepared by replacing tissue fluid with a curable polyester resin. Historically, brain slices (4-8mm thick) have been produced using the flat chamber technique. In previous years, P35 was used to prepare head slices (de Boer-van et al., 1993) and more recently, P40 polyester resin is being used for production of brain as well as body slices (Latorre et al., 2004). The P35 and P40 methods are named respectively for the Biodur™ polyester resins P35 and P40 used for their respective processes. The polyester plastination technique utilizes the basic principle of the classic plastination technique (von Hagens, 1979a; 1986; von Hagens et al., 1987. All water and a small portion of the lipid are removed from the slices and replaced with the curable polyester resin.

The Biodur™ P35 resin was developed in the late 1980's and after more than two decades, P35 still remains the gold standard for production of premier brain slices. In the mid 1990's, the P40 resin was introduced as a more user friendly method. A new comer to the polyester methodology and products is Hoffen P45 polyester resin. All of these resins utilize similar methodology with impregnation and casting between glass plates being the common theme among the polyester resin technology.

Classically, the P35-procedure produces opaque, thin (4, 6 or 8mm) brain slices while Epoxy resin (E12) produces semitransparent slices because much of the fat tissue is cleared with methylene chloride or acetone. However, thinner slices may be prepared and produced. Exquisite differentiation of white and gray matter is the premier quality of the Biodur™ P35 process.

The steps in polyester plastination are classic: specimen preparation, cold acetone dehydration, impregnation and finally curing (von Hagens, 1979b; 1986; Weber, 1992; Weber and Henry, 1992; Weiglein, 1996). However, an additional step of casting in a glass mold (flat-chamber) is added. Each step must be carried

out properly to assure a high quality plastinated slice. Chemicals used in polyester-plastination include:

- Acetone
- Polyester resin

The Biodur™ products for polyester plastination are:

- P35: Polyester resin
- A9: Catalyst

Materials and methods - Brain slices

The basic steps of plastination include: Specimen preparation, Dehydration, Impregnation and Curing.

Specimen preparation

The fresh brain specimen is harvested and fixed using 10% formalin, with no other additives, for three to six weeks (Riepertinger, 1988). As with most fixation methods, perfusion is the ideal method to achieve the best fixation. Fixation is necessary to yield the brain firm enough for slicing on a "deli"/meat slicer, as well as, to decrease any potential biohazard risk that could be associated with routine handling or slicing of tissue (Smith and Holladay, 2001). Specimens that have been fixed using formalin which contained other chemical additives should be used cautiously as the additives may cause adverse reactions with the resin.

Specimen preparation equipment:

- Deli/meat slicer
- Grids
- Grid/specimen basket

To aid orientation of the brain and to assure true transverse, horizontal or coronal slices and to keep the various pieces of the slice together as a single unit, the brain may be embedded in 20% gelatin and then sliced (Fig. 1) (Weber and Henry, 1992).

Slicing: A proposed plane of section of the brain is determined and the brain is cut into two portions with a brain (large) knife. One piece is placed into cold water and the other on the deli/meat slicer. Slices are prepared with the slicer at a determined thickness (Fig. 2). Slice thicknesses of 4, 6 or 8mm may be preferred since standard gasket sizes for these thicknesses are conveniently available from Biodur™. A produced slice is placed on a filter paper and then onto a metal or plastic (acetone resistant) grid (Fig. 1). The grids with their slices are stacked and submerged in a water bath. The stacked grids with their slices should be tied together in bundles or placed into a basket for ease of transfer from one acetone bath to another and subsequently into the resin-immersion baths and impregnation bath (Fig. 3).

Flushing & Precooling: The basket of slices is placed in cool running tap water overnight to rinse the formalin from the slices. Flushing may be extended for two days. After flushing, the slices are placed in fresh tap water and cooled to 4°C.

Specimen dehydration

The recommended dehydration procedure for plastination is freeze substitution in -25°C acetone (von Hagens, 1986; Tiedemann and Ivic-Matijas, 1988; Brown et al., 2002). Shrinkage is minimal when cold acetone is used. Ethanol dehydration promotes excess shrinkage of most tissue and especially brain tissue. Therefore, it should not be used for processing brain tissue.

Dehydration equipment:

- Deep freezer made explosion proof/safe
- Acetonometer
- Specimen/slice basket
- Chemical resistant acetone reservoirs

For all plastination techniques, acetone is ideal and serves both as the dehydration agent and intermediary solvent because acetone readily mixes with the resins used during plastination. It also has a high vapor pressure which allows maximal extraction from the brain slices. The stack of brain slices in the basket or bundle is removed briefly from the water and the excess water is allowed to drip off. The drained slices are submerged in the first cold (-25°C) acetone bath. Only this first acetone bath may be less than 100% purity (>90%). An estimate of acetone volume for one human brain is 25 L. The slices should be tilted a few degrees as they enter the acetone bath and agitated after submersion to remove trapped air bubbles. After 2 days, the basket of slices is moved to the second cold acetone (100%) bath of a similar volume. Caution: Dehydrated brain slices become brittle and break easily - Handle with CARE! After two days and stirring the acetone, the purity of acetone is checked with an acetonometer (Fig. 4). Acetone temperature must match the temperature calibration of the acetonometer. Most acetonometers are calibrated at +20°C, +15°C or -10°C. Therefore acetone must be warmed or cooled to match the calibrated temperature before measuring. If the acetone concentration is more than 98%, dehydration is considered complete. If more than 2% water remains, a third cold dehydration bath must be used to complete dehydration. Degreasing of brain slices is not done. Degreasing would cause excess shrinkage of the slices. When dehydration is complete, the slices are ready to be immersed into the resin-mixture.

Specimen impregnation

Immersion into resin-reaction mixture: The dehydrated brain slices will be transferred into an immersion bath in preparation for impregnation.

Day 1: An *immersion-mixture* of resin and catalyst is prepared. The ratio of resin (P35) to catalyst (A9) is **100:2 (P35- 100 pbw:A9- 2 pbw)**. These components

must be mixed thoroughly. The P35 process may be carried out in the deep freezer (-15°C), the refrigerator (+4°C) or at room temperature. The P35/A9 immersion-mixture *(bath #1)* is precooled to the chosen impregnation temperature. The basket or bundle of brain slices and grids are submerged into this precooled P35/A9 impregnation-mixture for one day. Caution: Immersion and impregnation baths must be kept in the dark to prevent the reaction-mixture from hardening (*UVA light is a catalyst). Note: This first immersion bath must be discarded after this use.

Day 2: *Immersion bath #2* of P35:A9 (100:2), mixed as bath #1, is prepared and mixed thoroughly. The slices are transferred from immersion bath 1 into bath 2. This second immersion bath may be used as the 1st immersion bath for the next group of slices.

Day 3: The slices are transferred into the well-mixed *impregnation reaction-mixture* which also has the same ratio of P35:A9 (100:2) as the two previous immersion baths. Allow the slices to equilibrate in the resin-mix and then forced impregnation is ready to begin. Impregnation may be carried out at either room temperature or in the cold.

Impregnation equipment:

- Vacuum chamber with a transparent lid
- Vacuum pump: oil preferred, dry may work
- Vacuum tubing and fine adjustment needle-valves
- Vacuum gauge
- Bennert mercury or digital manometer
- Specimen basket

Preparing the impregnation-mixture: The polyester impregnation bath is prepared by mixing Biodur™ P35 resin with A9 catalyst (100:2).

Forced Impregnation of brain slices: Prior to submersion of the slices into the fresh Biodur™ P35/A9 mixture (100:2), the vacuum pump is turned on and allowed to warm to working temperature for a few minutes. Once the pump has warmed, the slices in the impregnation-mixture are placed in the vacuum chamber at the desired temperature (room temperature or cold) and vacuum is applied. The vacuum chamber must be kept darkened. As the pressure is lowered, the acetone (because of its high vapor pressure) vaporizes/boils out of the slices leaving a tissue void into which the polyester resin-mix enters. Frequent regulation of the rate of evacuation is necessary to keep the impregnation boiling rate proper (rapid boil). Resin level must be observed and more reaction-mixture is added if the level drops and exposes the top slices. Pressure is slowly decreased to 10-12mm Hg at room temperature of 1-2mm Hg in the cold over a 24 to 30 hour period. Because the resin contains styrene, the listed lower limits of pressure, must be observed to prevent the extraction of styrene (von Hagens et al., 1987). When bubble production decreases considerably, impregnation is nearly complete, even though bubbling may not completely stop. Note: This impregnation-bath may be used as the 2nd immersion-bath for the next group of slices. After impregnation is complete, the chamber is returned to atmospheric pressure and the box containing the slices is removed and kept in a dark environment.

Curing or hardening the resin

UVA light serves as the catalyst for this Biodur™ polyester resin. The impregnated slices are placed between two glass plates to produce a smooth surface for the specimen.

Curing equipment:

- UVA lights
- 3-5mm (1/8in.) tempered (safety) glass
- 2mm (1/16in.) window glass
- Silicone gasket and 5-9cm spacer
- Large fold back clamps
- 1mm wire with small hooked tip

Preparing Glass/Casting Chambers for brain slices:

Each casting (flat) chamber is prepared from four appropriate sized pieces of glass. Two pieces are of safety (tempered) glass (3-5mm for strength) and two of float [window (2mm/ 1/16in for flexibility)]. Glass size of 35cm x 45cm is appropriate for human brain slices. Silicone gasket and fold-back (folder) clamps complete the necessities. The gasket length needs to be similar to the length of the perimeter of the glass. Both the top outer and the outer bottom glass of the chamber are to be the sheets of safety (tempered) glass. They are each lined with one inner sheet of float (window) glass. The intended top end of each pair of glasses is joined and sealed using masking tape (Fig. 5). This stabilizes the paired glasses and prevents resin from getting between them. The bottom end is secured with two smaller fold-back clamps. One set of glass plates will be placed on an assembly stand (block of Styrofoam or box) with the float glass facing up. The float glass is now ready to accept an impregnated brain slice.

The slices in their impregnation-mix are removed from the vacuum chamber. An individual slice with its grid is removed from the resin and allowed to drain briefly. The slice is placed on the float glass of the double glass plate unit that is resting on the assembly stand (Fig. 6) A silicone gasket (6mm for a 4mm slice) is placed 2cm from the bottom edge and its length is divided 2/3 to 1/3 from center of the bottom of the glass (Figs. 7, 8). This will allow the longer end to pass across the top of the glass and finally close the entire unit. Later the ends of the gasket will continue up the

sides lying on the float glass 2cm from the edge in preparation to seal the casting chamber. Place a 6mm spacer at the top. Next the second set of plates is placed on top of the slice, spacer and gasket with the float glass facing toward the specimen, thus forming the top of the chamber (Fig. 9). Remove the small fold back clamps. The gasket between the top and bottom is compressed by large fold-back clamps that are placed over the bottom and both sides of the chamber. Carefully align the clamping edge over the gasket to assure the best seal possible. The top end remains open with the excess gasket hanging out on each side. The clamps secure and seal the two sets of double glass plates with the gasket on three sides so that the flat chamber can be handled as one unit. Next, fold the bottom clamp handles onto the glass and stand the flat chamber containing the slice vertically on its bottom end with the open top directed upward (Fig. 10). The flat (casting) chamber containing the specimen is filled with the fresh well-mixed P35/A9 mixture (100:2). For filling the standard size chamber (35 x 45 cm), 700 ml of the resin-mixture is needed. A flat funnel, made by cutting off the corner of a plastic sleeve or flat plastic bag, is used to fill the chamber with a fresh mixture of P35/A9 (100:2) (Fig. 11). Air bubbles are poured into the chamber as the resin is introduced. Allow bubbles to rise to the surface. A small wooden wedge inserted into the opened top, to spread the glass, will aid trapped bubbles to rise to the surface. Some bubbles will cling to the surface of the slice and must be encouraged to rise. A 1mm wire may be used to tease or encourage a bubble to the surface. Leaning the chamber slowly from side to side will encourage trapped bubbles to rise. Check both sides of the flat chamber for bubbles. Use the wire to center the slice in the chamber. Remove the wedge and close the top of the chamber with the remaining length of the gasket and fold-back clamps (Fig. 12).

Light Curing: After casting, the double glass chambers are exposed to UVA-light to initiate curing (Figs. 13-15). Exposure time is 45 minutes depending on the wattage and the distance of the UVA lamps. Typically four 40W UVA lights (tubes) are used. Two are placed above and two below at a distance of 35 cm from the flat chamber. Now it is necessary to fold the handles of the fold-back clamps away from the glass to avoid marking the curing resin. During light exposure, it is necessary to cool the chambers on both sides either by ventilator (fan) or by blowing compressed air over both sides of the double glass chamber (Figs. 13-15). **Caution:** Cooling is important because the UVA-light commences an exothermic reaction that will harm the specimens if they are not cooled. To prevent cracking of the P35 slices and glass during light curing it is also recommended to use low watt UVA-lamps (40 watt).

Heat Curing: Following light curing, the double glass chambers are placed in a in a well-ventilated 40°C oven for 4-5 days. The clamp handles can be folded over the glass to save space in the oven. Heat finishes the curing of the resin into the depths of the specimen slice. At the end of day 4, turn off the oven. On day 5, the slices are removed from the oven to finish cooling. During cooling, cracking sounds may be heard as the cured resin separates from the glass. After cooling, the chamber is dismantled. The clamps, gasket, and paired glass plates from the top side are removed and then the remaining bottom plate set is removed. Occasionally, the float glass does not release by itself. To aid release of the glass plate from the slice, the tip of a scalpel blade may be used to score the length of the cured resin at the junction with the glass on one or more sides. After release, remove the plates and wrap the slice in light weight foil to prevent any uncured resin and debris from contacting the surface of the slice.

Day 1	Slice, flush and cool slices
Day 2	Immerse into first cold (-25°C) acetone bath (>90%). 1:10 specimen: acetone ratio recommended.
Day 4	Immerse into second cold (-25°C) acetone bath (100%). Check purity of bath #1.
Day 6	1st Immersion into +5°C or room temperature P35/A9 resin-mixture.
Day 7	2nd Immersion into +5°C or room temperature P35/A9 resin-mixture.
Day 8	Impregnate in fresh +5°C or room temperature P35/A9 resin-mix.
Day 9	Cast and UVA light cure at room temperature for 45 minutes.
Day 10	Heat cure in 40°C (105°F) oven.
Day 14	Open flat chamber, cover slice with foil, saw and sand.

Table 1: General protocol for P35 technique - Polyester brain slices (4-8mm).

Finishing:

After curing, release and wrapping is complete, the excess cured resin is trimmed to the desired size and shape on a band saw. The edges may be smoothed using a belt sander or sanding paper/cloth. The gasket and glass can be cleaned in a dishwasher or using hot water and an enzymatic detergent.

Results

The finished P35 sections are durable, opaque, easy to orient, and may be correlated with radiographic, CT and MR images (Figs. 16-18). In many cases,

orientation of nerve fibers can be observed. Blood vessels, substantia nigra and many nuclear structures may be seen. These slices are of the highest aesthetic value because of the smooth surface finish, clarity of the resin and enhanced contrast of white and gray matter. There is no odor associated the finished slices.

Discussion

Flat chambers are set upright and filled with the polyester resin mixture (P35/A9). Hence, the plastinated slices are incorporated into the sheets of the plastination resin as the resin cures and are not merely embedded. Yet the cover of resin is thin enough to avoid refractive distortion that is proprietary to wet specimen mounts. The glass yields a mirror smooth finish to each side of the slice. The specimens show marked delineation of white and gray matter and are durable. Using this technique, slices of the brain are more detailed, more durable and easier to handle than those produced with other techniques. The sheets containing the slices are cured initially using ultraviolet light from UVA lamps and finished in a 40°C oven.

Production of these superb slices is very labor intense and seems to be an expensive methodology. As well, three aliquots of resin are necessary due to the short working time of the immersion and impregnation-mixtures. This necessitates that a large quantity of resin-mix will be discarded unless it is planned to run concurrently several appropriate size lots of slices. Even with excellent planning it is difficult to fit these plans into the week's schedule. Likely it will involve a portion of the weekend and the next week. However, much of the equipment is found in an active plastination laboratory. Therefore, there is usually not a large capital outlay for equipment for production of polyester slices.

P35 resin is more viscous than P40 resin which makes it more difficult for bubbles to rise and be extracted. Clear glass is needed to visualize bubbles and aid extraction, as well as allow UVA light penetrate to commence resin curing. Window glass is not sturdy enough to prevent bowing of glass from the weight of resin and the slice. Therefore, hardened glass must be used to support the unit and prevent bowing and assure a flat slice. Tempered glass often is too firm to release from the cured resin. Therefore, the window glass must be placed next to the resin/slice to accommodate the shrinking resin as it cures.

Technically light cure could be bypassed. However, heat cure alone reduces the resin's viscosity and would allow the specimen to move in the chamber. Meanwhile, light cure solidifies the resin surrounding the slice and fixes its location within the resin pool. The excess resin surrounding the cured slice allows a series of slices to be uniformly trimmed to the same dimension for enhanced presentation. Or this surrounding area may be used for labeling. The main advantage of the P35 sheet plastination method is the superb clarity of nervous tissue and the enhanced differentiation of white and gray matter.

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Product distribution:

Biodur Products, Drs. Gunther von Hagens and Angelina Whalley, Rathausstrasse 11, 69126 Heidelberg, Germany. www.biodur.de, Ph: (49) 6221 3311-11, Fax: (49) 6221 3311-12.

Figure 1. Human brain slices embedded in gelatin - P35 technique.

Figure 2. Preparing 2mm brain slices with a deli slicer for dehydration, impregnation, casting and curing for plastination via the P35 technique.

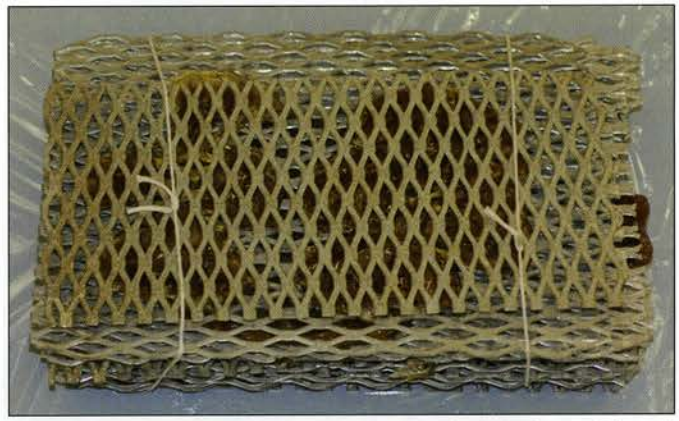

Figure 3. Human brain slices embedded in gelatin, positioned on grids, bundled, and tied for transfer into acetone and resin-mix.

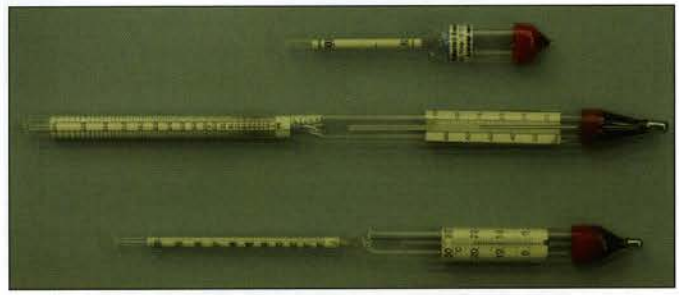

Figure 4. Assortment of acetonometers for checking acetone purity.

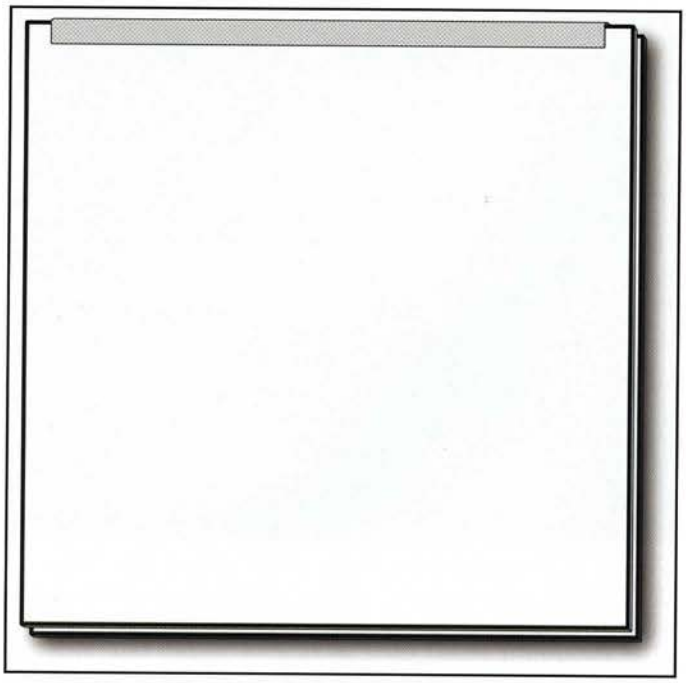

Figure 5. A prepared and taped double glass plate set ready to accept impregnated slice.

Figure 6. Impregnated human brain slice positioned on the float glass of the bottom set of taped, double glass plates ready for gasket placement.

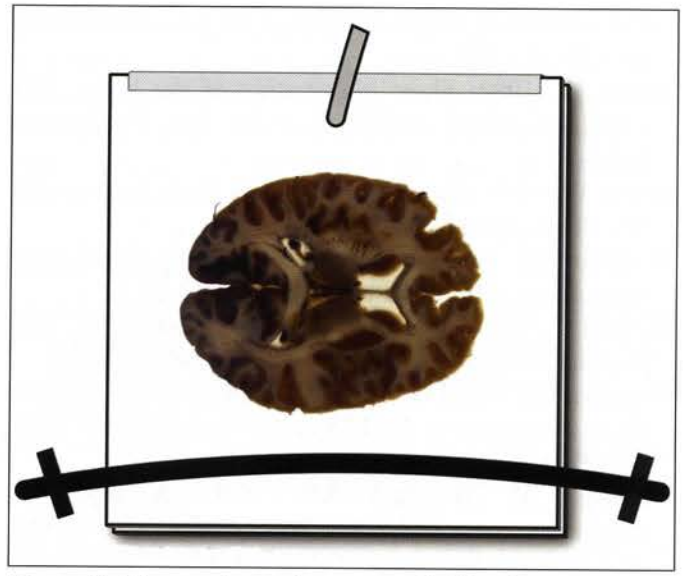

Figure 7. Slice, gasket and spacer on double plate set.

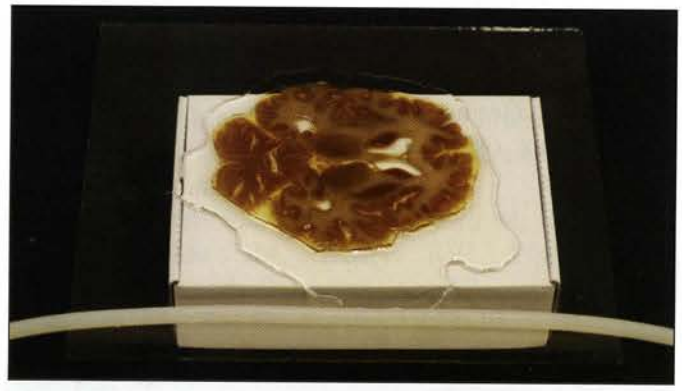

Figure 8. P35 impregnated slice on the float plate.

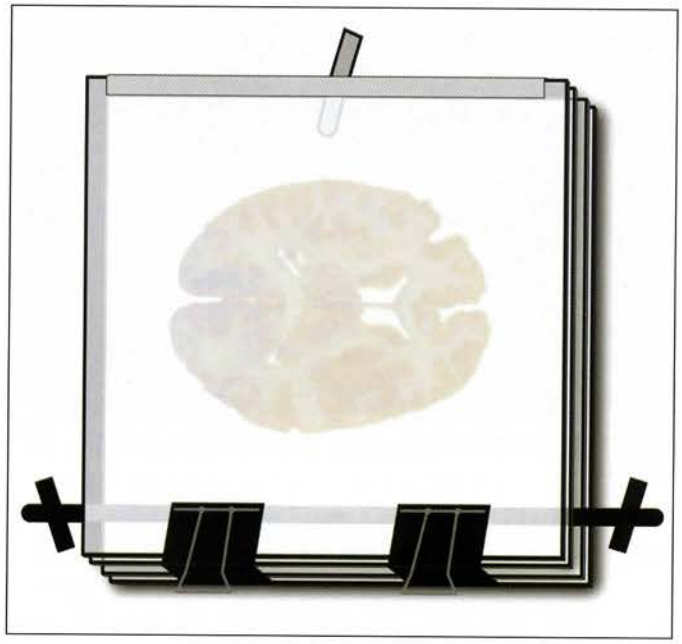

Figure 9. Top double glass plate in position over slice, gasket and spacer. Bottom clamps in place.

Figure 10. Flat chamber assembled, containing an impregnated slice, ready to be filled with resin-mix.

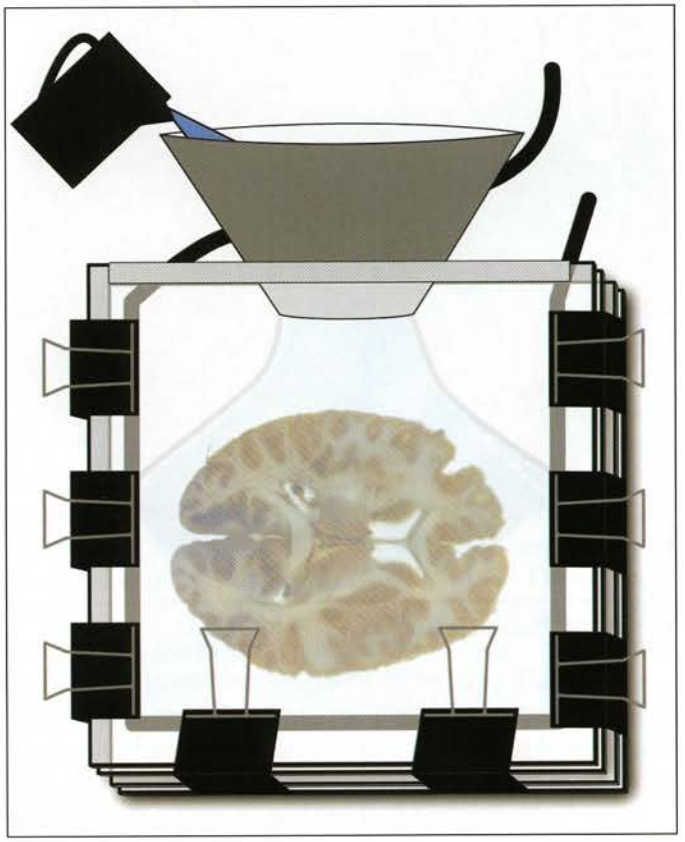

Figure 11. Filling a double glass chamber with P35 resin-mix.

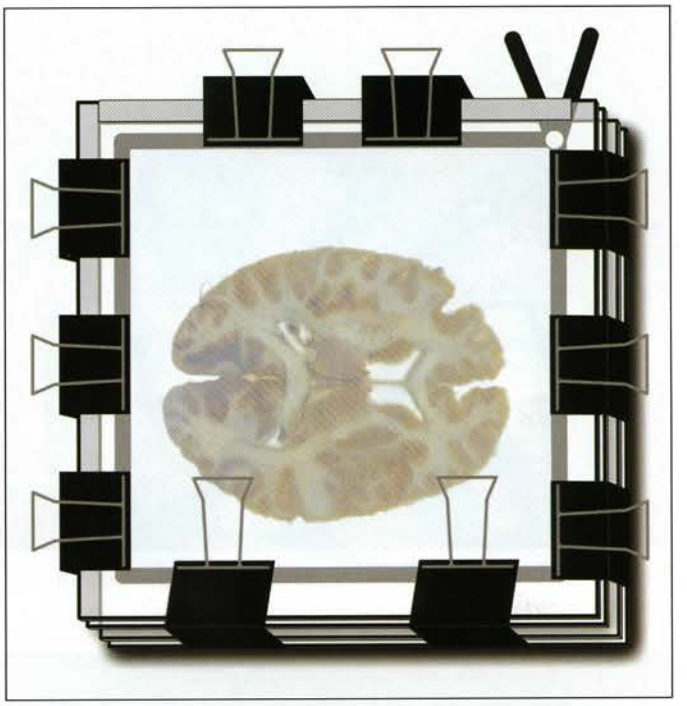

Figure 12. Closure of top gasket after filling with P35.

Figure 13. Exposing impregnated slice in flat chamber to UVA light with ventilator (fan).

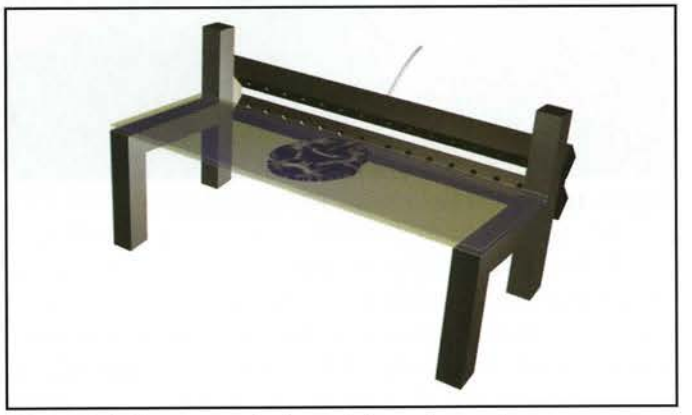

Figure 14. Compressed air cooled, curing rack.

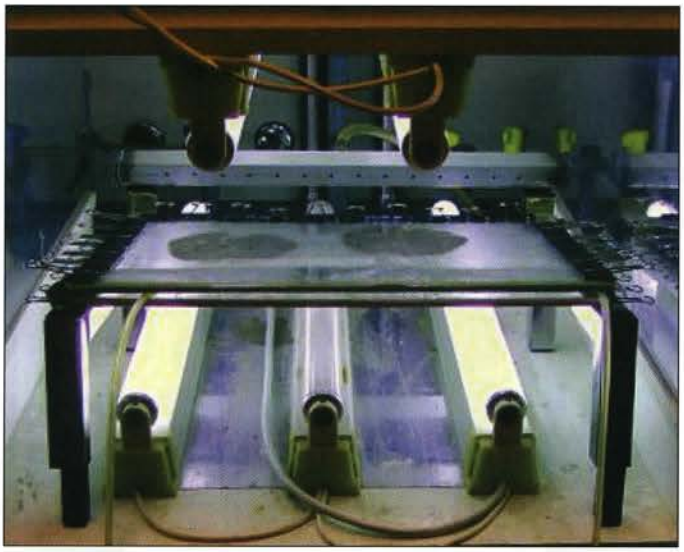

Figure 15. UVA light exposure rack with compressed air cooling apparatus.

Figure 17. Human brain slice after cutting and trimming excess cured resin from its perimeter - P35 technique.

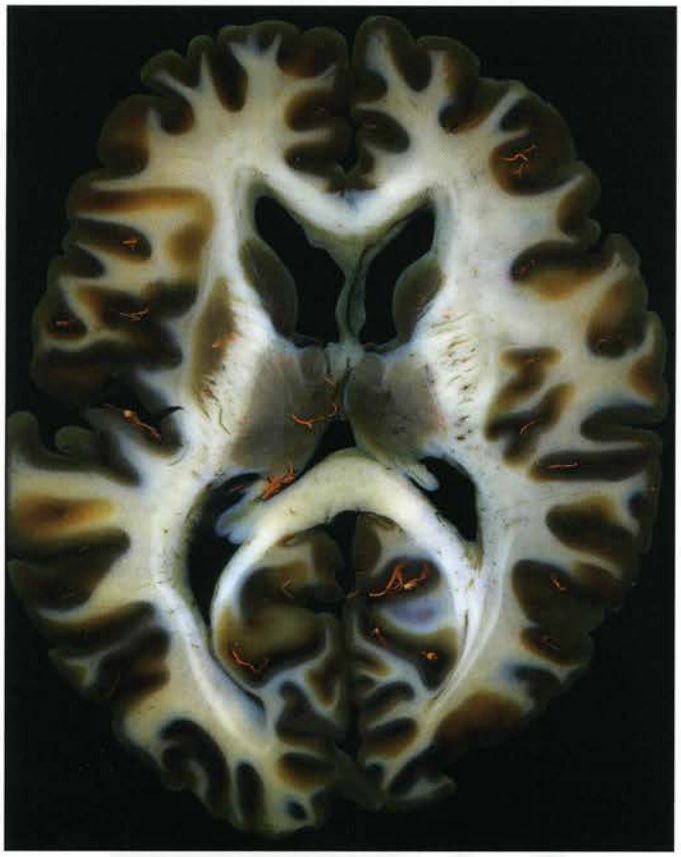

Figure 16. Human brain slice - P35 technique.

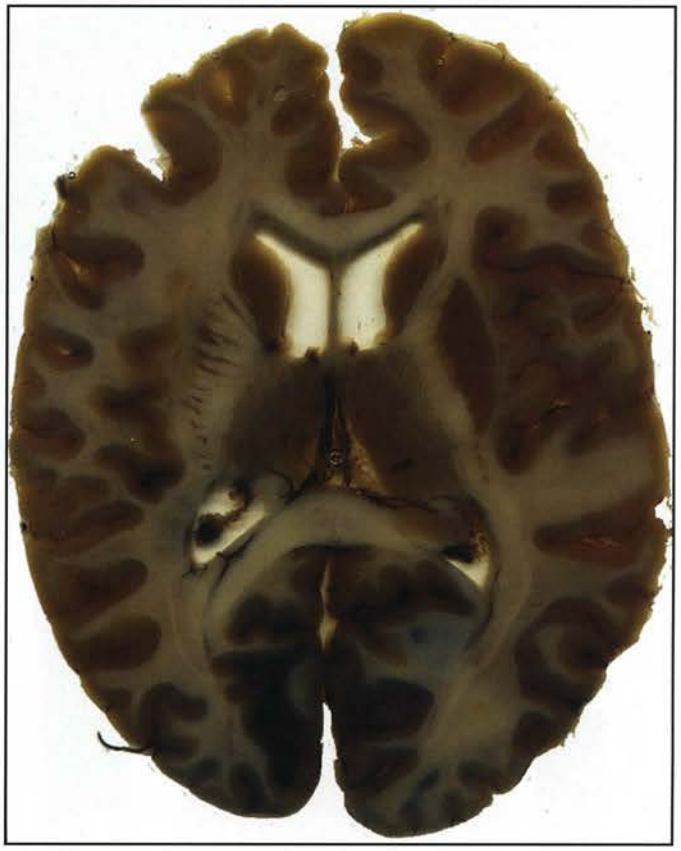

Figure 18. Human brain slice - P35 technique.

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Polyester Plastination of Biological Tissue: P40 Technique for Brain Slices

R.W. HENRY[1*] and R. LATORRE[2]

[1]*Department of Comparative Medicine, College of Veterinary Medicine, University of Tennessee, Knoxville, TN, USA.*

[2]*Anatomía y Embryología, Facultad de Veterinaria, Universidad de Murcia, Murcia, Españia, Europe.*

[*]*Correspondence to: Telephone:* (865) 974 - 5822; *Fax:* (865) 974 - 5640; *E-mail:* rhenry@utk.edu

Abstract: Plastination has become the gold standard for preservation of biological tissues. Plastination supports many disciplines: anatomy, art, biology, clinics and medicine. The Biodur™ P40 technique produces 2-3mm translucent brain slices which display vivid sectional anatomy. These unique slices are excellent teaching aids especially when used with modern diagnostic images: Computed tomography, Magnetic resonance and Ultrasound. The P40 technique was developed as a less complex method for presentation of brain slices.

Key words: **plastination; polyester method; brain slices; polyester resin; P40; A4**

Introduction

Polyester impregnated slices of brain tissue, cast in glass-flat chambers, have been used in teaching and research (Barnett, 1997, Henry and Weiglein, 1999, Sora et al., 1999; Weiglein and Feigl, 2001, Latorre et al., 2002). In the late 1980's, the Biodur™ P35 resin was developed for production of 4-8mm slices of brains (von Hagens, 1990). Biodur™ P35 brain slices remain the gold standard for brain slice production even today. The P40 resin was introduced in the mid 1990's as a less complicated process (von Hagens, 1994). The P40 technique accommodates production of thinner slices. White/gray differentiation with P40 is less exquisite than white and gray matter differentiation of brain slices produced by the P35 process. Polyester plastination utilizes the basic principals of the classic plastination techniques (von Hagens, 1979; 1986; von Hagens et al., 1987). The central premise of plastination is removal of tissue fluid and its replacement with a curable polyester resin. Prepared dual manuscripts define the production of P40 brain slices as well as P40 body slices.

Chemicals used in polyester-plastination include:

- Acetone
- Polyester resin

The Biodur™ products for polyester plastination are:

- P40: Epoxy resin
- A4: Activator

Materials and methods

The basic steps of plastination include specimen preparation, dehydration, impregnation and curing.

Specimen preparation

Note for production of Biodur™ P40 brain slices, the basic steps of specimen preparation, slicing and dehydration are similar to the Biodur™ P35 Technique. Please refer to specimen preparation section of "The P35 Technique" for a more detailed description.

A brain specimen is harvested and fixed for six weeks using 10% formalin or a long term-fixed brain may be used (Riepertinger, 1988). A well-fixed firm brain is desirable for slicing on a meat slicer, as well as, to diminish biohazard risk associated with routine handling or slicing of biological tissue (Smith and Holladay, 2001). Specimens that have been embalmed with chemicals other than formalin should be avoided.

Well-fixed brains are divided and sliced on a deli/meat slicer with its thickness set for 2-3mm slices (Fig. 1).

Specimen preparation equipment:

- Deli/meat slicer
- Grids
- Grid/specimen basket

Slicing: Determine the plane of section of the brain. Divide the brain into two portions. Place one piece in a cold water bath and the other on the meat slicer. Slices are prepared at a determined thickness (2-3mm) (Figs. 2, 3). The slices are inspected, cleaned if necessary with running water and placed on an acetone resistant grid (metal or plastic). The slices on their grids are stacked and submerged in a water bath. It is beneficial to tie the stacked grids with their brain slices together in a bundle or place into a specimen basket for ease of transfer into the acetone bath or into the resin-impregnation bath.

Flushing & Precooling: The bundle of slices is placed in cool running tap water for one or two days to rinse debris and formalin from the slices. After flushing, the bundle of slices remains in water and cooled to +4°C over night.

Specimen dehydration

Freeze substitution in -25°C acetone is the recommended procedure for dehydration of all plastinated specimens (von Hagens, 1986; Tiedemann and Ivic-Matijas, 1988; Brown et al., 2002). Dehydration using the classic ethanol method promotes excess shrinkage of most tissues and especially brain tissue. Never use alcohol to dehydrate brains for plastination. Freeze substitution produces negligible shrinkage. It is established that for all plastination techniques, acetone is the best dehydrating agent but also is a good intermediary solvent for impregnation. As well, acetone readily mixes with the resins used during plastination and has a high vapor pressure that aids its extraction from the brain slices.

Dehydration equipment:

- Acetonometer
- Specimen/slice basket
- Chemical resistant acetone reservoirs

The bundle of grids and brain slices is raised from the water to allow the excess water to drip off. After draining, the slices are placed into the **first** cold acetone bath (-25°C). Only this first acetone bath may be less than 100% purity but >90%. Entering the acetone bath, the bundle of slices is tilted to allow trapped air bubbles to float to the surface. In two days, the slices are submerged in the **second** cold acetone bath of a similar volume but of 100% purity. Use caution, dehydrated brain slices are brittle and easy to break. Check the purity of bath #2 after two days. Acetone purity is checked using an acetonometer in an aliquot of well-mixed acetone. Acetonometers are temperature specific (Fig. 4). Most are calibrated at +15°C, +20°C, or -10°C. The acetone temperature must match the temperature calibration of the acetonometer. This means that the acetone must be cooled or warmed to the calibrated temperature before measuring and recording purity. If the acetone concentration is more than 98%, dehydration is considered complete. A third acetone bath is used if more than 2% water remains. Degreasing of brain slices is not done because excess shrinkage of the slices would result. With the completion of dehydration, the bundle of slices is ready to be immersed into the impregnation/resin-mixture.

Specimen impregnation

Impregnation equipment:

- Vacuum chamber with a transparent lid
- Vacuum pump (Oil type pump is recommended, dry pump may be used)
- Vacuum tubing and fine adjustment needle-valves
- Vacuum gauge
- Bennert mercury or digital manometer
- Specimen basket

Preparing the impregnation-mixture: The polyester impregnation bath can be P40 resin with no additives or a Combination of P40 resin plus 1-2% A4 (activator). **Resin must be kept in dark.

Immersion of brain slices into P40 resin: The dehydrated brain slices are transferred into and submerged in a room temperature or +5°C impregnation bath of P40 resin. This resin must be kept darkened as UVA light serves as the catalyst for this polyester. This polyester impregnation bath can be of P40 resin alone or a Combination of P40 resin plus 1-2% A4 (activator). It is okay to commence impregnation at this time. However, consider letting the slices sit in the P40 resin overnight. This allows the slices, acetone and resin to equilibrate before commencing forced impregnation. It is necessary to keep the vacuum chamber covered from light. Impregnation may be carried out at room temperature or in the cold (+5°C).

Forced Impregnation of brain slices: After submersion of the slices into the P40 resin, the vacuum pump is turned on and allowed to warm to working temperature for a few minutes. Once the pump has warmed, the slices in the impregnation-mixture are placed in the vacuum chamber at the desired temperature (room temperature or cold) and vacuum is applied. The vacuum chamber is kept dark. As the pressure is lowered, trapped air is evacuated first. Many small bubbles can be seen rising to the resin surface. The rate of evacuation (lowering of pressure) is regulated frequently to keep the pressure decreasing to reach the vapor pressure of the solvent which is temperature

dependent: Acetone 22cm/10in Hg or MeCl 43cm/ 17in Hg @ 25°C; Acetone 8cm/3in Hg or MeCl 18cm/4in Hg @ 5°C. After the air has been evacuated and when the vapor pressure of acetone is reached, the acetone (because of its high vapor pressure) vaporizes/boils out of the slices leaving a tissue void into which the polyester resin or resin-mix enters (Henry, 2005a; 2005b). As the solvent exits from the brain slices, a tissue void remains within the brain, into which the polyester resin enters. The vaporized solvent is pumped out through the pump exhaust. Pressure is continuously decreased, by incrementally closing the needle valves, to 5cm Hg for room temperature and 2cm Hg for cold impregnation by the end of the first day of impregnation. The vacuum pump remains working overnight. More resin is be added if necessary to keep the slices submerged. Concluding the 30 hour period, the next day pressure is incrementally decreased to 1cm Hg at room temperature or 1-2mm Hg in the cold room. When the low pressure level is reached and maintained for a few hours and bubble formation has slowed, impregnation is complete. Because the resin contains styrene, these lower limits of pressure, must be observed to prevent the extraction of styrene (von Hagens et al., 1987).

After impregnation is complete, the reservoir is returned to atmospheric pressure. The box containing the slices and the P40 impregnation bath maybe removed and kept in a dark environment.

Curing or hardening the resin

UVA light serves as the catalyst for this polyester resin. The impregnated slices are placed between two glass plates to obtain a smooth surface and a thicker more durable specimen.

Curing equipment:

- UVA lights
- 2mm (1/16in.) window glass
- Silicone gasket and 5cm spacer
- Large fold back clamps
- Ball bearings and magnet
- 1mm wire with small hooked tip
- Biodur™ gasket seal (HS 80)
- 18ga (1.2mm) hypodermic needle

Preparing Glass/Casting Chambers for brain slices: There are two methodologies for constructing casting chambers: a. Building the **chamber around a slice** and b. Building the chamber for later insertion of slice into the chamber. Insertion of the slice after construction of the glass chamber is recommended only for slices that do not have multiple parts. Each casting (flat) chamber is prepared from two same, appropriate sized pieces of window (2mm or 1/16in) glass, silicone gasket and large fold-back (folder) clamps. Gasket length is to be similar to the length of the perimeter of the glass. Both top and bottom walls of the chamber each consist of one sheet of window (float) glass. To prepare to accept a slice, one glass plate is placed on an assembly stand (block of Styrofoam or box) and construction begins as follows:

a. Building flat chamber around a brain slice: One slice on its grid is removed from the resin and excess resin is allowed to drain briefly before placing the brain slice onto the center of the glass which is setting on the assembly stand (Fig. 5). A silicone gasket (5mm for a 3mm slice) is placed 2cm from the bottom edge of the glass (Fig. 6) and its length is divided 2/3 to 1/3 from the center point of the glass. This will allow enough length to pass across the top of the glass and close the top of the flat chamber at the top corner. Later the ends of the gasket will continue up the sides lying 2cm from the edge in preparation to seal the casting chamber. A 5cm spacer, made of gasket, is placed near the top of the glass (Fig. 6). The spacer will support the top edge of the top glass. The top glass plate is placed over the brain slice, spacer and gasket (Fig. 7). Fold-back clamps are placed along the perimeter of the bottom glass over laying the gasket. Care is taken to align the clamping edge of the clamp over the gasket which will assure the best seal possible. Both ends of the gasket are turned (90° angle) toward the top and positioned 2cm from the glass edge. Clamps are positioned directly over the gasket (Fig. 8). The spacer is removed and the top end of the flat chamber remains open with the excess gasket hanging from each side. The clamps secure and seal the glass plates with the gasket. Next, fold the bottom clamp handles onto the glass and stand the flat/glass chamber, containing the slice, vertically with the open top directed upward (Fig. 6). The flat (casting) chamber containing the specimen is filled with the fresh well-mixed P40 resin or P40/A4 (100:1-2) resin/activator-mixture. A flat funnel is used to fill the chamber with P40 (Fig. 9). Air bubbles are poured into the chamber and must be allowed to rise to the resin surface. Some bubbles will attach to the surface of the slice and must be encouraged to rise. A small wooden wedge inserted in the opened top will spread the glass and aid bubble removal. A 1mm wire may be used to tease or encourage a bubble to float to the resin surface. Tipping the chamber from side to side will encourage trapped bubbles to rise. Check both sides for bubbles. Use the wire to center the slice in the chamber. Close the top of the chamber with the remaining longer length of the gasket and fold back clamps (Fig. 10). Make sure the resin level totally fills the chamber. Remove any air bubbles lying against the top gasket. Air interferes with the curing of the P40 resin. At the top corner of the

chamber, seal the junction of the bent ends of the gasket with Biodur™ gasket seal (HS 80).

b. Build chamber for later insertion of a brain slice: Position a glass on the assembly stand. An appropriate sized silicone gasket is placed 2cm from the bottom edge and its length is divided 2/3 to 1/3 at the midpoint of the glass (Fig. 11). The longer end will be used to close the top after insertion of the slice and filling with resin. Later the ends of the gasket will continue up the sides lying 2cm from the edge in preparation to seal the casting chamber. A spacer placed near the top of the glass will support the top glass. The top plate is placed on top of the spacer and gasket (Fig. 12). Fold-back clamps are placed along the perimeter of the bottom of the glasses over-lying the gasket. Align the clamping edge over the gasket to assure the best seal possible. Both ends of the gasket are turned (at a 90° angle) toward the top and positioned 2cm from the edge of the glass. Clamps are positioned directly over the gasket. Remove the spacer and the top is to remain open with the excess gasket hanging to each side. The positioned clamps secure and seal the glass plates with the gasket along the bottom and the sides. Next, fold the bottom clamp handles onto the glass and stand the flat/glass chamber vertically with the open top directed upward (Fig. 13). A brain slice is inserted through the top opening and the flat (casting) chamber now containing the specimen is filled with the fresh well-mixed P40 resin or the P40/A4 (100:1-2) mix (Fig. 9). Allow air bubbles to rise to the resin surface in the chamber. Trapped bubbles must be encouraged to rise to the top by using a small wooden wedge inserted in the opened top and a 1mm wire, along with tipping the chamber from side to side. After bubble removal, close the top of the chamber with the remaining longer length of the gasket and fold back clamps making sure to release any bubbles trapped against the top gasket. Seal the junction of the bent ends of the gasket with Biodur™ gasket seal (HS 80) (Fig. 14). Inspect for large air bubbles trapped along the upper gasket. Remove bubble by insertion of an 18ga (1.2mm) hypodermic needle or 1mm wire between the glass and the gasket (Fig. 14).

Tip 1: Insert one or two 3mm ball bearings into the chamber before closing the top. The bearings will be pushed by a magnet to centrally position and align the brain slice to your specification. This mechanism is also helpful if the slice moves while you transport the slice and position it between the UVA lights. After the slice is centered, park the ball bearings in one corner of the chamber. They will be removed when the excess cured resin is cut from the slice.

Time saver: Once the art of casting is understood, the top gasket does not need to be closed and sealed. If the resin level is 2-3cm below the top of the glass and yet adequately covering the slice, the chamber with its bottom and sides sealed and containing the slice and surrounding P40, may be laid at a 15° angle from the horizontal between the UVA light (catalyst) source and curing will proceed. Along the open edge, a 0.5mm thickness of resin will not cure. However, it will be cut off during regular trimming of the slice.

Light Curing: After casting, to initiate and finalize curing, the sealed glass chambers, filled with resin, slice centered and fold back clamp handles turned off of the glass, are exposed to UVA-light (Fig. 15). A minimum of one hour is recommended for curing, depending on the wattage and distance of the UVA lamps. Typically four - 40 watt UVA light bulbs (tubes) are used. Two lights are placed above and two below the glass chamber at a distance of 35 cm from the flat chamber. During light exposure, it is necessary to cool the chambers on both sides either by a ventilator (fan) or blowing compressed air over both sides of the glass chamber. Caution: Cooling is important because the catalyst (UVA-light) commences an exothermic reaction that will harm the specimen if not cooled and too much heat is built up. To prevent cracking of the glass and/or damage to the slice from excess heat during light curing, monitor the glass surface temperature and shut off the UVA-lamps if the glass temperature rises to 30°C. Continue constant cooling even when light is off and slice is cool.

Tip 2. Cooling can be enhanced by curing in a walk in cold room (3°C) or out of doors when the ambient temperature is less than room temperature Ventilators are still recommended to move the heat of the exothermic reaction away from the glass.

Tip 3. Use natural daylight out of doors (shadow is recommended) as an effective way to cure the cast slices. It is not necessary to close or seal the top, provided the chamber is laid at an appropriate angle from horizontal to prevent leakage. The chamber must be turned every 15 minutes to assure uniform exposure to the catalyst (UV light of the sun, but in the shade). Also for best aesthetics, the position of the slice may shift and need to be centered using the ball bearing and magnet or wire. Ventilation is recommended to remove the heat from the glass. The casting chamber may be allowed to set up vertically. However, the slice tends to sink and rest on the bottom gasket. Hence no resin margin is available when trimming the slices for final display.

If A4 activator is used in the impregnation-mix, the slices also need to be kept in the dark and cool. They need to be cast in a few days after impregnation. Specimens impregnated only in P40 resin may be stored

Figure 1. Deli/meat slicer.

Figure 2. Slicing 2.5mm brain slices.

Figure 3. Slicing 2.5mm brain slices.

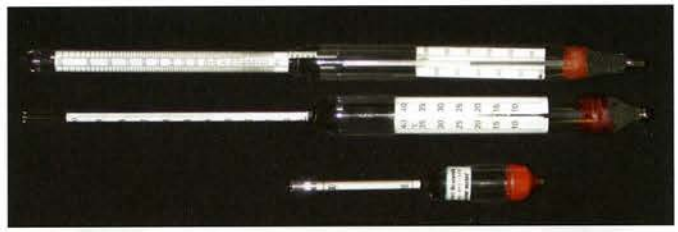

Figure 4. Acetonometers with various temperature requirements.

Figure 5. Building flat chamber around the slice. Brain slice centered on bottom glass plate.

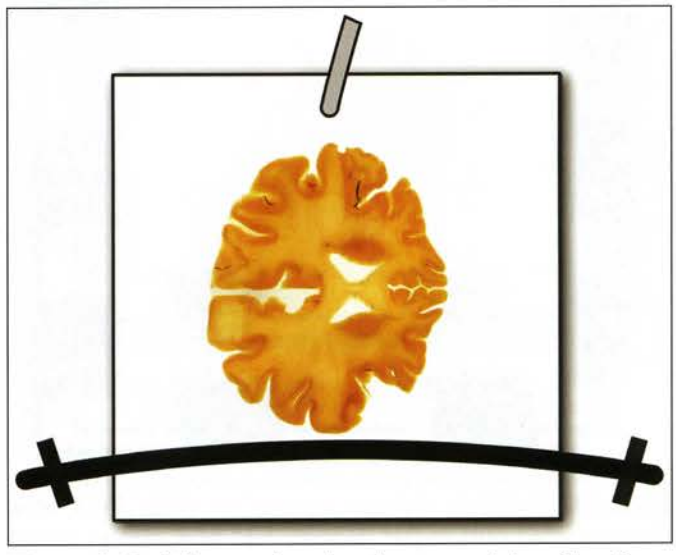

Figure 6. Building casting-chamber around the slice: 2mm glass, slice, spacer and gasket.

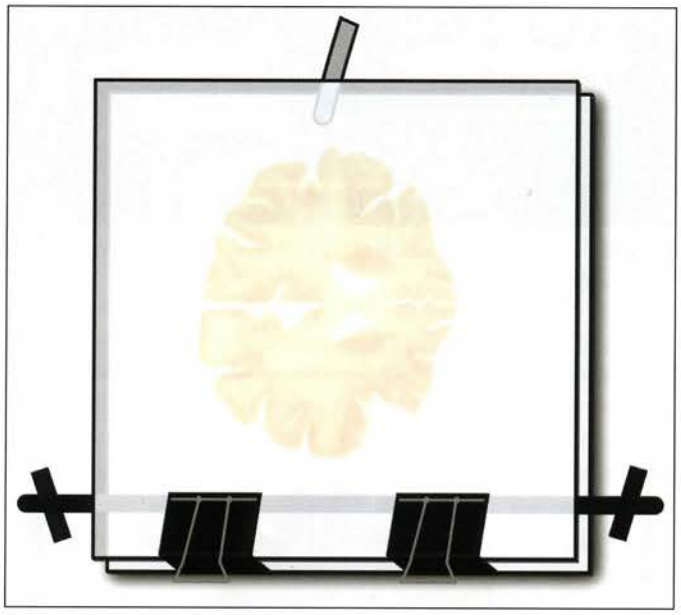

Figure 7. Building chamber around the slice: Top glass positioned.

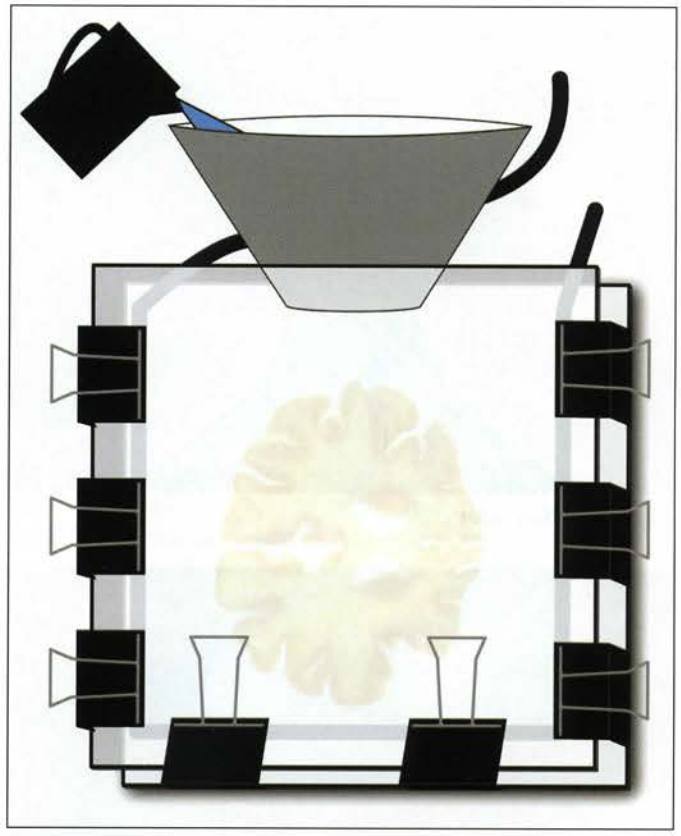

Figure 9. A flat funnel is used to fill chamber with P40.

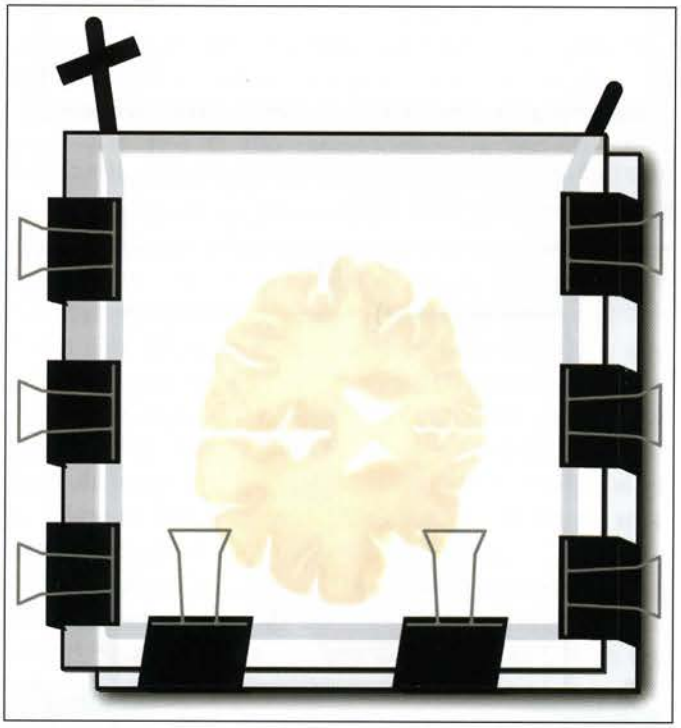

Figure 8. Building chamber around the slice: Fold back clamps placed over gasket. Bottom fold back clamps turned onto the glass.

Figure 10. Building chamber around the slice: Close and seal top gasket.

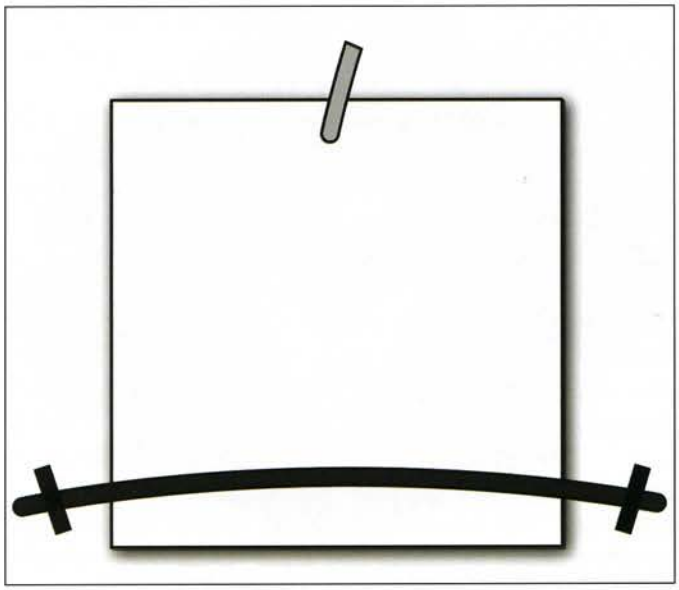

Figure 11. Building the casting-chamber for later insertion of the slice: 2mm glass, spacer and gasket.

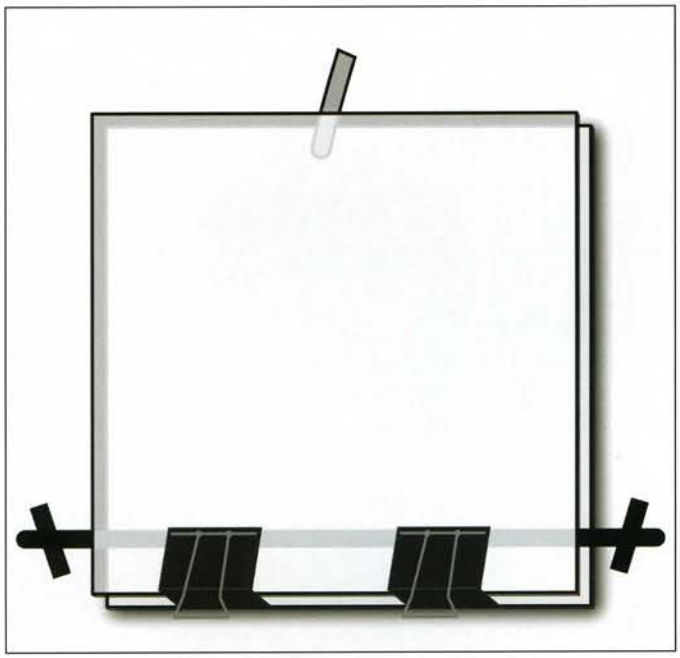

Figure 12. Building chamber for later insertion of the slice: Positioning top glass.

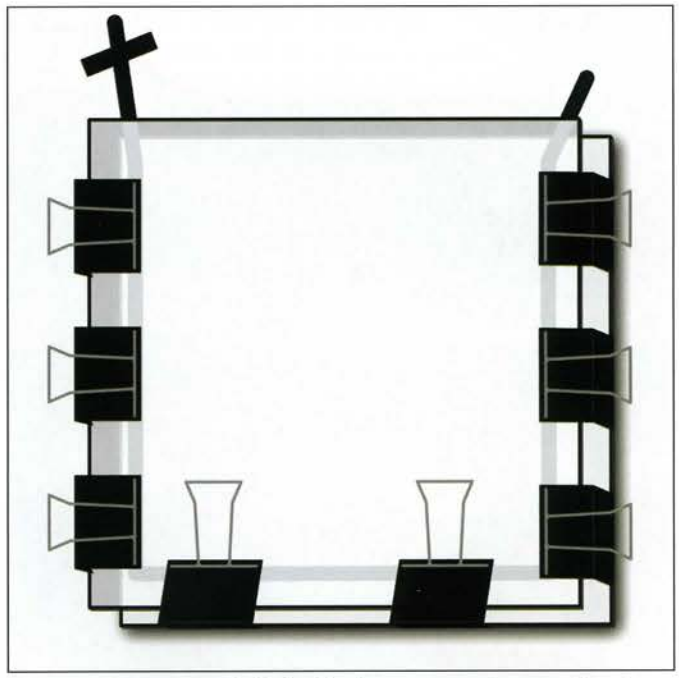

Figure 13. Bottom fold back clamps turned onto the glass. Chamber ready to receive the brain slice.

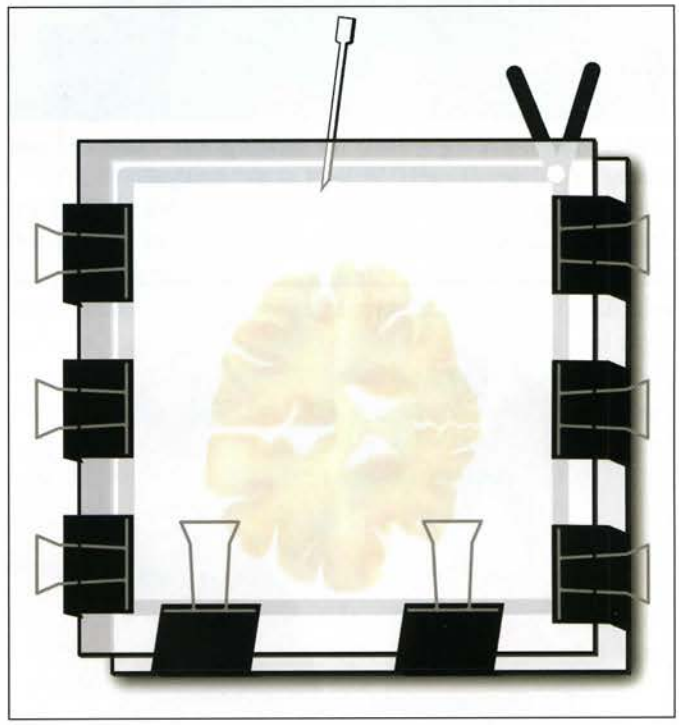

Figure 14. Air bubble removal with hypodermic needle.

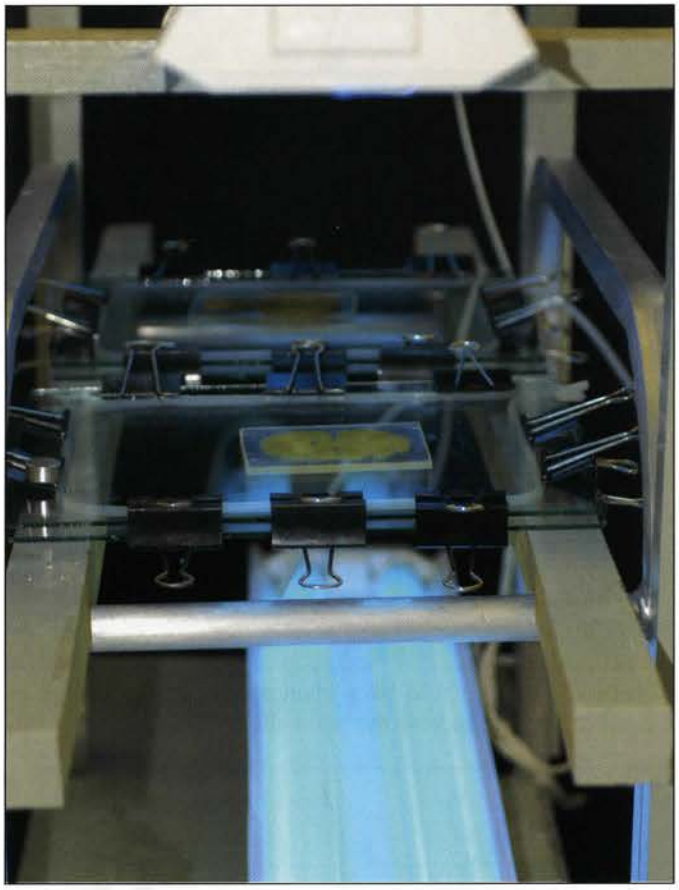

Figure 15. UVA light rack for holding flat chambers and curing cast slices using UVA light as the catalyst.

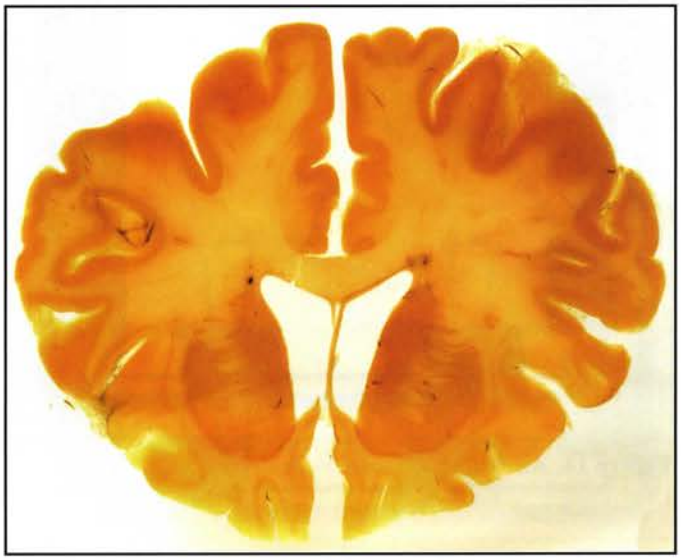

Figure 16. Cured equine brain slice plastinated using the P40 technique.

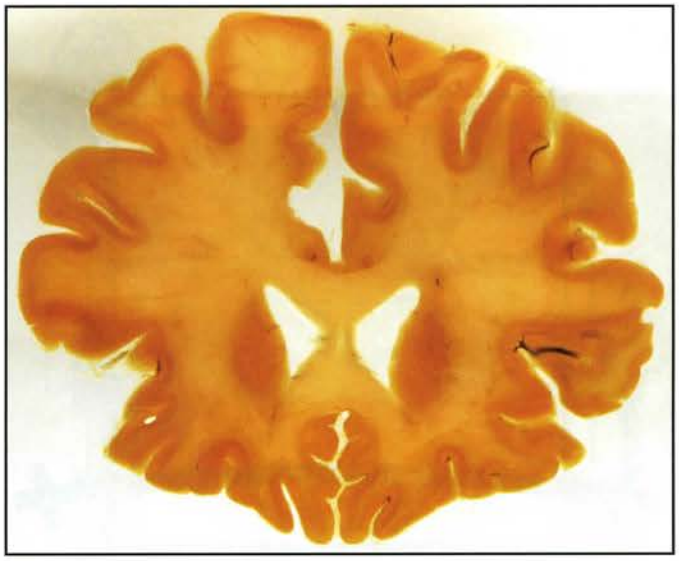

Figure 17. Cured P40 plastinated equine brain slice.

Figure 18. Cured P40 brain slices wrapped in foil. Note position of ball bearings in corner of the cast slice.

a long time in the dark before casting.

Finishing:

After curing or during cooling, cracking sounds may be heard as the cured resin releases from the glass. After assurance of curing, the chamber is dismantled. The clamps and gasket are removed and then the casting chamber glass. Occasionally, the glass does not release by itself. To aid release of the glass, the tip of a scalpel blade is scored along the length of the junction of the glass with the resin on one or more sides. After release, remove the plates and wrap the specimen in light weight foil (plastic wrap) to prevent any uncured resin and debris from contacting and/or smearing on the surface of the slice.

After curing, release and wrapping is complete, the excess cured resin is trimmed on a band saw. The edges may be smoothed using a belt sander or sanding paper/ cloth. The gasket and glass can be cleaned in a dishwasher and/or with hot water and an enzymatic detergent.

Day 1	Slice, rinse and cool slices
Day 2	Immerse in first cold (-25°C) acetone bath (>90%) acetone. 1:10 specimen:acetone ratio.
Day 4	Immerse in second cold acetone bath (100%), Check purity of bath #1.
Day 6a	Check purity of bath #2. If <98% - Immerse into P40 or P40+A4-mix.
Day 6b	Check purity of bath #2. If >98%, Immerse into third cold acetone bath (100%).
Day 7	Impregnate with the chosen P40 bath.
Day 9	Cast and UVA light cure (bulbs or indirect natural light)
Day 10	Open flat chamber, cover slice with foil, saw and sand.

Table 1: General protocol for P40 Technique - Polyester brain slices (2-3mm).

Results

The finished P40 sections are durable, semi-transparent, easy to orient, and correlate nicely with radiographs, CT and MR images (Figs. 16-18). They are tremendous teaching aids. White-gray matter differentiation as well as optical quality is good.

Discussion

The brain slice in the sealed flat chamber and after laid horizontal between the UVA lights does not migrate. It will stay centered in the resin. Non-sealed flat-chambers which are cured upright or at an angle allow specimens to move/sink and special attention must be given at the appropriate time (before curing) to keep the slices centered in the resin. Using P40 resin or P40 mixed with A4 has been equally successful at producing desirable specimens. The step of addition of A4 was noted and recommended a few years after release of the P40 resin for slice production because of potential problems with curing, possibly caused from inadequate fixation (Henry, 1998). If brain tissue is well-fixed and impregnation is complete, the use of A4 may not be necessary. The advantage of not using A4 is the indefinite pot-life of the P40 impregnation bath if kept cool and in the dark. Without A4 additive, the same impregnation bath has been used for over five years. As well, without the additive, impregnated slices may be stored for years before casting (when timing is more convenient or if slices are needed for demonstration).

The impregnated brain slice is surrounded by polyester resin or mixture (P40/A4) while it is curing. Hence, the plastinated slices are incorporated into a single cured sheet of the resin. They are not merely embedded in the resin. The specimens show good delineation of white and gray matter and are durable, as well as demonstrate good anatomical detail for comparison with images of modern imaging modalities (Latorre et al., 2002). Using this technique, slices of the brain are more detailed, more durable and easier to handle than those produced by other techniques.

One main advantage of the P40 sheet plastination method is the decreased volume of resin used: Two-thirds less, because there is no separate immersion bath as with the P35 technique. As well, the P40 impregnation bath may be reused many times. Also, the process is not complicated and less equipment and time are needed to run the P40 technique. In addition to brain slices, body slices may be produced using this basic technique (Latorre et al., 2004). P40 slices are excellent teaching aids for teaching sectional anatomy (Latorre et al., 2001; Henry, 2005c).

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Product distribution:

Biodur Products, Drs. Gunther von Hagens and Angelina Whalley, Rathausstrasse 11, 69126 Heidelberg, Germany. www.biodur.de, Ph: (49) 6221 3311-11, Fax: (49) 6221 3311-12.

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Polyester Plastination of Biological Tissue: P40 Technique for Body Slices

R. LATORRE[1*] and R.W. HENRY[2]

[1]*Anatomía y Embryología, Facultad de Veterinaria, Universidad de Murcia, Murcia, Españia, Europe.*
[2]*Department of Comparative Medicine, College of Veterinary Medicine, University of Tennessee, 2407 River Drive, Knoxville, TN, 37996-4543, USA.*

[*]*Correspondence to: Telephone:* (34) 986 364 697; *Fax:* (34) 986 364 147; *E-mail:* latorre@um.es

Abstract: Plastination has developed into the gold standard for preservation of biological tissues and specimens. Plastination has been applied to many areas: anatomy, art, biology, clinical medicine, surgery and research. Semi-transparent 2-3mm body slices which display sectional anatomy in conjunction with its anatomical surroundings are produced using the P40 polyester technique. These manufactured slices are excellent aids for teaching medicine and to help understand modern diagnostic images: Computed tomography, Magnetic resonance and Ultra sound. Originally the polyester technique was developed for presentation of brain tissue. However in this decade, polyester is being used with a variety of tissues.

Key words: **plastination; polyester method; body slices; polyester resin; P40; A4**

Introduction

Polyester impregnated slices of biological tissue, manufactured in glass-flat chambers, are a useful format for anatomical study or research (Barnett, 1997; Henry and Weiglein, 1999; Sora et al., 1999; Weiglein and Feigl, 2001; Latorre et al., 2002). Classically, 4-8mm slices of nervous tissue were the norm (von Hagens, 1990). The P35 resin was developed in the late 1980's and remains the gold standard for brain slice production. In the mid 1990's, the P40 resin was introduced as a less complex technique. Currently, thinner slices are prepared with P40 (von Hagens, 1994). However, their white/gray differentiation is second alone to the exquisite differentiation of white and gray matter of brain slices of the P35 process.

Polyester plastination utilizes the same basic principals of the classic silicone plastination techniques (von Hagens, 1979a; 1979b; 1986; von Hagens et al., 1987; Henry and Nel, 1993). Tissue fluid is removed and is replaced with a curable polyester resin. More recently, P40 polyester resin is used also for body slices (Latorre et al., 2002; 2004). P35 has been used to prepare head slices (de Boer-van et al., 1993). P40 also was used to plastinate a nerve plexus (Sora, 1998). P45 resin is a new comer to the polyester methodology (Gao et al., 2006) and uses heat as its catalyst. These resins utilize similar methodology with impregnation and casting between glass plates their unique features. Dual manuscripts present the production of P40 body slices as well as the production of P40 brain slices.

Chemicals used in polyester-plastination include:

- Acetone
- Methylene chloride
- Polyester resin

The **Biodur™ products for polyester plastination** are:
P40: Epoxy resin
A4: Activator

Materials and methods - Body slices

The basic steps of plastination are: specimen

preparation, dehydration, impregnation and curing.

Specimen preparation

Note for production of P40 body slices, the steps of specimen preparation, slicing and dehydration are the same as for the E12 epoxy technique. Please refer to this section of "E12 epoxy technique" for more details.

Specimen preparation equipment:

- Band saw
- Grids
- Grid/specimen basket

Specimen preparation - body slices:

The selected specimen is positioned for proper anatomical alignment and frozen preferably in an ultra-cold deep freezer for two days (longer for larger specimens). Fresh tissue is preferred. Tissue should be fixed in formalin if deemed a potential for exposure to biohazards that may be associated with routine handling and sawing of biological tissues (Smith and Holladay, 2001). Preservation of tissue color is the best reason not to use formalin. Specimens covered with hair should have their hair clipped. Ultra-cold freezing is necessary to yield the best tissue slices. Formalin fixative solutions containing other chemical additives should be thoroughly rinsed to deplete the tissue of any chemicals that potentially may react adversely with the resin.

Slicing: The slices of the frozen specimen are cut on a band saw (Fig. 1). A guide stop is necessary for cutting uniform sections. Cooling the stop and/or saw table with ice prevents premature thawing of the specimen, as well as the slices. The proposed plane of section of the specimen is determined and the end of the tissue block is squared for commencing sawing. 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 taking place. The guide stop is set for the desired specimen thickness (2-3mm) and serial sections are sawed. As slices are produced, they are placed on metal or plastic grids (acetone resistant) in preparation for saw dust removal (Fig. 2). Dust removal may be done by: Returning the slice on the grid into the freezer and scraping the dust with the knife blade in the cold environment; Submerging the frozen slice in the first dehydration bath and scraping the dust from the specimen while submerged; or Running a rapid, small stream of tap water across the surface of the slice while scraping the surface with a knife (Fig. 3). Caution should be used and not allow the slice to thaw, especially unfixed brain tissue. The grids with their cleaned slices are stacked in the cold acetone (-25°C) of the **first** acetone bath (Fig. 4). Slices must be submerged to prevent drying/freezer burn of the slices. The stacked grids with their slices should be tied together in a bundle (Fig. 5) or placed into a basket for convenience and ease of transfer from one acetone bath to another and into the impregnation resin.

Dehydration and degreasing of body slices

The recommended dehydration procedure for plastination is freeze substitution in -25°C acetone.

Dehydration equipment:

- Acetonometer
- Specimen/slice basket
- Chemical resistant acetone reservoirs

Shrinkage is minimal when cold acetone is used. For all plastination techniques, acetone is the ideal dehydrant and serves both as the dehydration and defatting agent, as well as the intermediary solvent. Also, acetone readily mixes with the resins used for plastination. The bundle of slices is submerged in the first cold acetone bath which may be less than 100% (>90%) purity. The slices should be tilted a few degrees as they enter the acetone bath and agitated after submersion to remove trapped air bubbles. Trapped air bubbles can cause drying of tissue which can show up on the finished slice. After the slices have been in the first cold acetone bath for 3 days, the stack of body slices in the basket or bundle is removed briefly from the first acetone bath. The excess acetone is allowed to drip off quickly to prevent surface drying. Then the slices are submerged in the second cold (-25°C) 100% acetone bath. **Caution:** Dehydrated slices, especially brain, become brittle and break easy - Handle with CARE! After two or three days in the second bath, the purity of acetone is checked with an acetonometer. The acetone bath must be stirred and the acetone temperature must match the temperature calibration of the acetonometer (+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. If the acetone concentration is more than 98%, dehydration is considered complete. However if more than 2% water remains (<98% acetone purity), a third dehydration bath must be used to complete dehydration. Degreasing of body slices is essential to obtain the best resolution of adjoining tissues. Degreasing should not be done on brain slices, degreasing will cause excess shrinkage of the slices.

Degreasing of body slices

Degreasing of body slices is accomplished by setting the dehydrated body slices out of the deep freezer into room temperature for the necessary time for degreasing to occur (1 - 3 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) (MeCl) for one or two days. Monitor degreasing in MeCl daily. When body slices are appropriately degreased, transfer the slices from their bath (acetone or MeCl) into the impregnation resin.

Specimen impregnation

Impregnation equipment:

- Vacuum chamber with a transparent lid
- Vacuum pump (oil type pump is preferred)
- Vacuum tubing and fine adjustment needle-valves
- Vacuum gauge
- Bennert mercury or digital manometer
- Specimen basket
- Polyester P40 resin

Preparing impregnation-mixture: The polyester impregnation bath can be P40 resin with no additives or a Combination of P40 resin plus 1-2% A4 (activator).

Immersion into P40 resin of body slices: The dehydrated and degreased body slices are transferred into and submerged in a room temperature impregnation bath which must be kept darkened since light serves as the catalyst for this polyester. This impregnation bath can consist of P40 resin alone or may be a combination of P40 resin plus 1-2% A4 (activator). The slices in the resin can be placed in the vacuum chamber and vacuum can be applied and impregnation started immediately. However, it is beneficial to allow the slices to sit in the P40 resin overnight and equilibrate before applying vacuum and commencing forced impregnation. It is necessary to keep the vacuum chamber covered from light. The polyester resin is activated by light. Impregnation may be carried out at room temperature or in the cold (+5°C).

Forced Impregnation of body slices: The vacuum pump is turned on the next morning and allowed to warm to working temperature. Once the pump has warmed, vacuum is applied. The vacuum chamber must be kept darkened. Frequent regulation of the rate of evacuation (lowering pressure) is necessary especially in the beginning to evacuate the air trapped in the resin. As the pressure is lowered, air will boil from the resin and be pumped off. When pressure is lowered to the vapor pressure of the solvent (Acetone 22cm/9in Hg, MeCl 43cm/17in Hg @ +25°C), solvent will begin to vaporize (Henry, 2005a; 2005b). It will exit from the slices leaving a tissue void into which the polyester resin enters. The volatilized solvent is pumped out through the exhaust of the pump. The goal should be to keep the impregnation rate at a moderate boil and pressure slowly decreasing over the rest of the day and night. Resin level must be monitored and more P40 added if the level drops and exposes the top slices. The pressure level by the end of the day should be around 5cm Hg for room temperature or 2cm Hg for cold room impregnation. The pump is allowed to pump over night. The next day pressure is decreased incrementally to 1cm Hg at room temperature or 1-2mm Hg in cold temperature over the conclusion of this 30 hour impregnation period. Monitor the rate of decrease by bubble production, keep a moderate boil. When these pressure levels are reached and maintained for a few hours, impregnation is complete. Because the resin contains styrene which is released at low pressure, the lower limits of pressure are suggested to prevent styrene release from the resin and damaging the vacuum pump (von Hagens et al., 1987). When bubble formation decreases considerably, impregnation is nearly complete, however bubbling may not completely stop.

After impregnation is complete, the chamber is returned to atmospheric pressure. The box containing the slices and the P40 impregnation bath is removed and kept in a dark environment.

Curing or hardening the resin

UVA light serves as the catalyst for this polyester resin. The impregnated slices are places between two glass plates to obtain smooth surfaces of the specimen.

Curing equipment:

- UVA lights
- 2mm (1/16in.) window glass
- Silicone gasket and 5cm spacer
- Large fold back clamps
- Ball bearings and magnet
- 1mm wire with small hooked tip
- Biodur™ gasket seal (HS 80)
- 18ga (1.2mm) hypodermic needle

Preparing Glass/Casting Chambers for brain slices: Two methodologies for constructing casting chambers are used: a. Building the chamber around a slice; b. Building the chamber for later insertion of a body slice into the chamber. Insertion of the body slice after construction is recommended only for slices that do not have multiple parts. Each casting (flat) chamber is prepared from two same and appropriate sized pieces of window (2mm or 1/16in) glass, silicone gasket and large fold-back (folder) clamps. The gasket length should be similar to the length of the perimeter of the glass. Both top and bottom sides of the chamber consist of one sheet of window (float) glass. To prepare to

accept a slice, one glass plate will be placed on an assembly stand (block of Styrofoam or box) and construction begun as follows:

a. Building flat chamber around a body slice: An individual slice on its grid is removed from the resin and excess resin allowed to drain briefly before placing the slice onto the center of the glass which is setting on the assembly stand. A silicone gasket (4mm for a 2mm slice) is placed on the glass, 2cm from the bottom edge (Fig. 6). The gasket length is divided 2/3 to 1/3 from the center point of the glass. This will allow enough length to pass across the top of the glass and close the top of the flat chamber. Later the ends of the gasket will continue up the sides lying 2cm from the edge in preparation to seal the casting chamber. A spacer is placed near the top of the glass which will support the top edge of the top glass. Next the top glass plate is placed on top of the slice, spacer and gasket (Fig. 7). Fold-back clamps are placed along the perimeter of the bottom glass over laying the gasket. Care is taken to align the clamping edge of the fold-back clamp over the gasket which will assure the best seal possible. Both ends of the gasket are turned (90° angle) toward the top and positioned 2cm from the edge of the glass. Clamps are positioned directly over the gasket as before. The top remains open with the excess gasket hanging from each side. The clamps secure and seal the glass plates with the gasket. Next, fold the bottom clamp handles onto the glass and stand the flat/glass chamber, containing the body slice, vertically with the open top directed upward. The flat (casting) chamber, containing the specimen, is filled with the fresh well-mixed **P40 resin** or **P40/A4 (100:1-2) mixture**. A flat funnel is used to fill the chamber with P40 (Fig. 8). Air bubbles are poured with the resin into the chamber and must be allowed to rise to the surface. Some bubbles will catch on the surface of the slice and must be encouraged to rise. A small wooden wedge inserted in the opened top will spread the glass and aid bubble removal. A 1mm wire may be used to tease or encourage a bubble to float to the surface. Leaning the chamber from side to side will encourage trapped bubbles to rise. Check both sides for bubbles. Use the wire to center the slice in the chamber. Close the top of the chamber with the remaining longer length of the gasket and fold back clamps. Make sure the resin level totally fills the chamber. Remove any air bubbles lying against the top gasket (Fig. 9). Air interferes with the curing of the P40 resin. Seal the junction of the bent ends of the gasket with a 4mm ball of Biodur gasket seal (HS 80). Inspect for large air bubbles trapped along the upper gasket. Remove bubbles by insertion of an 18ga (1.2mm) hypodermic needle between the glass and the gasket.

b. Build chamber for later insertion of a body slice: Position a glass on the assembly stand. An appropriate size and length silicone gasket is placed 2cm from the bottom edge and its length is divided 2/3 to 1/3 at the midpoint of the glass. The longer end will be used to close the top space after filling. Later the ends of the gasket will continue up the sides lying 2cm from the edge in preparation to seal the casting chamber. A spacer placed near the top of the glass will support the top glass. The top plate is placed on top of the spacer and gasket. Fold-back clamps are placed along the perimeter of the bottom of the glasses over-lying the gasket. Align the clamping edge over the gasket to assure the best seal possible. Both ends of the gasket are turned (90° angle) toward the top and positioned 2cm from the edge of the glass. Clamps are positioned directly over the gasket. The top remains open with the excess gasket hanging to each side. The positioned clamps now secure and seal the glass plates with the gasket along the bottom and the sides. Next, fold the bottom clamp handles onto the glass and stand the flat/glass chamber vertically with the open top directed upward. A body slice is inserted through the top opening and the flat (casting) chamber now containing the specimen is filled with the fresh well-mixed P40 resin or the P40/A4 (100:1-2) mix (Fig. 8). Allow air bubbles to rise to the surface. Trapped bubbles need to be encouraged to rise to the top by using a small wooden wedge in the opened top and a 1mm wire along with leaning the chamber from side to side. Close the top with the remaining longer length of the gasket and fold back clamps making sure to release any bubbles trapped against the top gasket (Fig. 9). Seal the junction of the bent ends of the gasket with a 4mm ball of Biodur gasket seal (HS 80). Inspect for large air bubbles trapped along the upper gasket. Remove bubble by insertion of an 18ga (1.2mm) hypodermic needle or 1mm wire between the glass and the gasket.

Tip 1: Insert one or two 3mm ball bearings into the chamber before closing the top. They can be used to centrally position and align the slice to your specification with the aid of a heavy duty magnet. This is also helpful if the slice moves while you transport and position it between the UVA lights.

Light Curing: After casting, to initiate and finalize curing, the sealed glass chambers filled with resin and a slice (Fig. 10) are exposed to UVA-light. A minimum of one hour is recommended for curing, depending on the wattage and distance of the UVA lamps. Typically four - 40 watt UVA light bulbs (tubes) are used. Two are placed above and two below the glass chamber at a distance of 35cm from the flat chamber. During light exposure, it is necessary to cool the chambers on both

sides either by ventilator (fan) or blowing compressed air over both sides of the glass chamber. Caution: Cooling is important because the catalyst (UVA-light) commences an exothermic reaction that will harm the specimen if not cooled. To prevent cracking of the glass and/or damage to the slice from excess heat during light curing, monitor the glass surface temperature and shut off the UVA-lamps if the glass temperature rises to 30°C while continuing constant cooling.

Time saver: Once the art of casting is understood, the top gasket does not need to be closed and sealed. If the resin level is 2-3cm below the top of the glass, yet adequately covering the slice, the chamber with its bottom and sides sealed and containing the slice and surrounding P40 may be laid at a 15° angle from horizontal between the UVA light (catalyst) source and curing will proceed as anticipated. Along the open edge a 0.5mm thickness of resin will not cure, but it will be cut off during regular trimming of the slice.

Tip 2. Cooling can be enhanced by curing in a walk in cold room (+3°C) or out of doors when the ambient temperature is less than room temperature. Ventilators are still recommended to move the heat of the exothermic reaction away from the glass.

Tip 3. Use natural daylight outside (shadow is recommended) as an effective way to cure the cast slices. It is not necessary to close or seal the top, provided the chamber is laid at an appropriate angle from horizontal to prevent leakage. The chamber must be turned at 15 minute intervals to assure uniform exposure to the catalyst (UV light of the sun, but in the shade). Also for best aesthetics, the position of the slice may shift and need to be centered using the ball bearing and magnet or wire. Ventilation is necessary to remove the heat from the glass. The casting chamber may be allowed to set up vertically. However, the slice tends to sink and rest on the bottom gasket. Hence no resin margin is available when trimming the slices for final display.

Finishing: After curing and during cooling, cracking sounds may be heard as the cured resin releases from the glass. After assurance of curing, the chamber is dismantled. The clamps and gasket are removed along with the casting chamber glass. Occasionally, the glass will not release by itself. To aid release of the glass, the tip of a scalpel blade is used to score along the length of the junction of the glass with the resin on one or more sides. After release, remove the plates and wrap the specimen in light weight foil (plastic wrap) to prevent any uncured resin and debris from contacting the surface of the slice.

After curing, release and wrapping is complete, the excess cured resin is trimmed using a band saw. The edges may be smoothed using a belt sander or sanding paper/cloth. Glass and gasket are cleaned in a dishwasher or using hot water and an enzymatic detergent.

Day 0	Prepare and freeze specimen in anatomical position.
Day 1	Slice and clean saw dust from slices. Immerse in first cold (-25°C) acetone bath (>90%) acetone. 1:10 specimen: acetone ratio.
Day 4	Immerse in second cold acetone bath (100%)
Day 7	Check acetone purity, Bring out to room temperature to degrease.
Day 7	Immerse in third acetone bath (100%) if warranted.
Day 14	Check quality of degreasing.
Day 14 or Day x	When degreasing is finished, Immerse into P40 resin or P40+A4 mix.
Day x +1	Impregnate with P40 or P40/A9-mix.
Day x +3	Cast and UVA or natural light cure
Day x +4	Open flat chamber, cover slice with foil, saw and sand.

Table 1: General protocol for P40 - Polyester body slices (2-3mm).

Results

The finished P40 sections are durable and semi-transparent. They are excellent sectional anatomy aids and may be used as visual aids to study radiographs, CT and MR images (Figs. 11, 12).

Discussion

The slices in the sealed flat chambers laid horizontal between the UVA lights do not migrate and will stay centered in the resin during curing. Non-sealed flat-chambers which are cured upright or at an angle allow specimens to move/sink and special attention must be given at the appropriate time to keep the slices centered in the resin. Using P40 resin or P40 mixed with A4 have been equally successful at producing desirable specimens. The A4 addition is recommended to assure curing of the resin and prevent curing artifacts and prevent problems with curing from inadequate fixation (Henry, 1998). The advantage of not using A4 is the indefinite pot-life of the P40 impregnation bath if kept cool and in the dark. Without A4 additive, the same impregnation bath has been used for five years. As well, without the additive, impregnated slices may be stored for years before casting (when timing is more convenient or when slices are needed for demonstration).

Figure 1. Slicing frozen tissue block on a band saw.

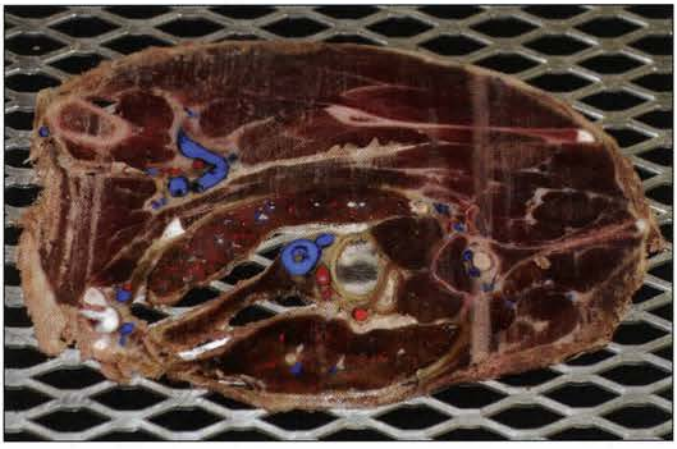

Figure 2. Saw dust covered slice on grid.

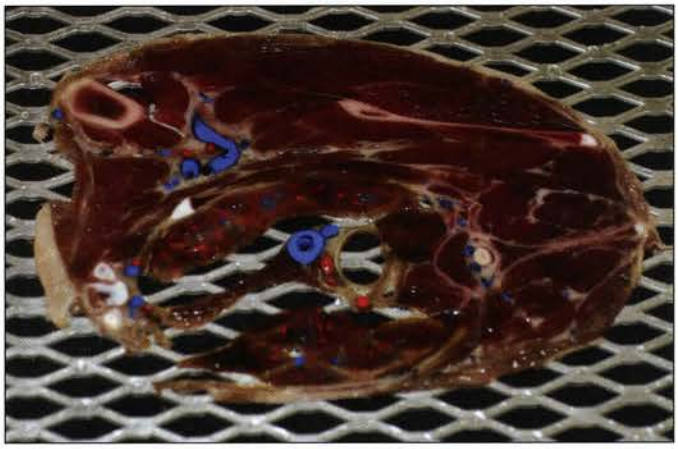

Figure 3. Slice after saw dust removal.

Figure 4. Bundled slices in first acetone bath.

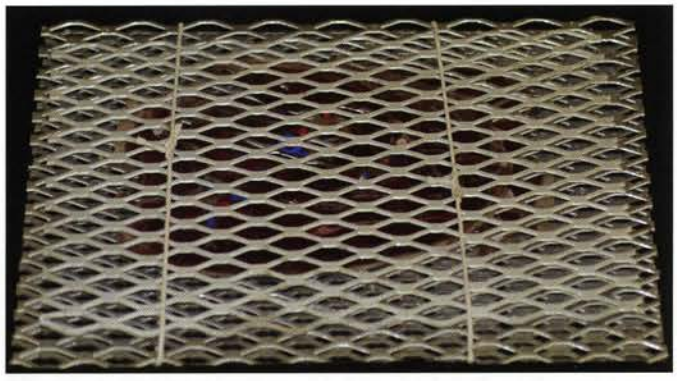

Figure 5. Bundled slices.

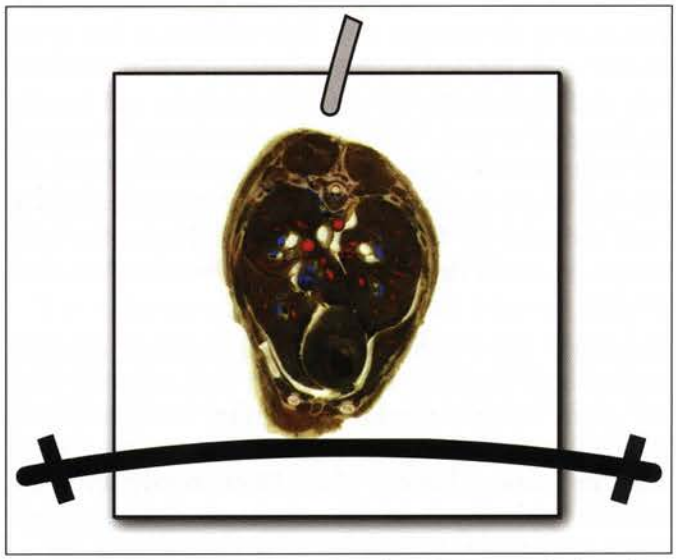

Figure 6. Building the casting-chamber around the slice: 2mm glass, slice, spacer and gasket.

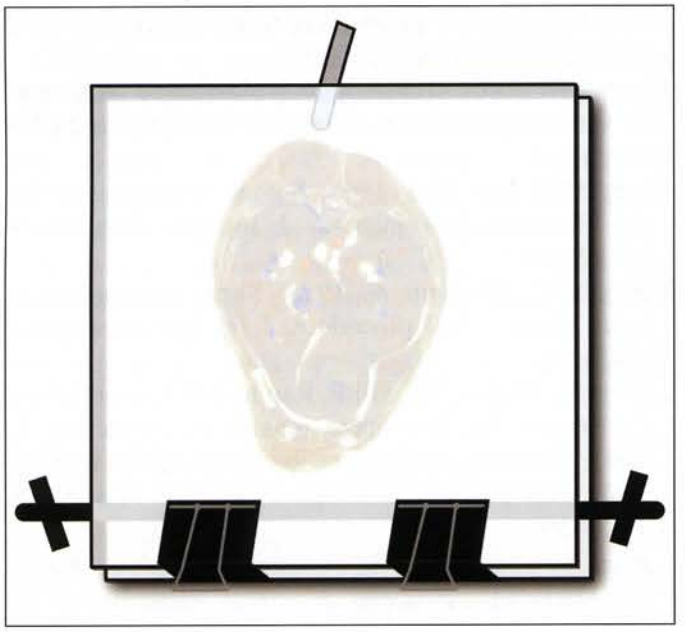

Figure 7. Building chamber: Positioning top glass.

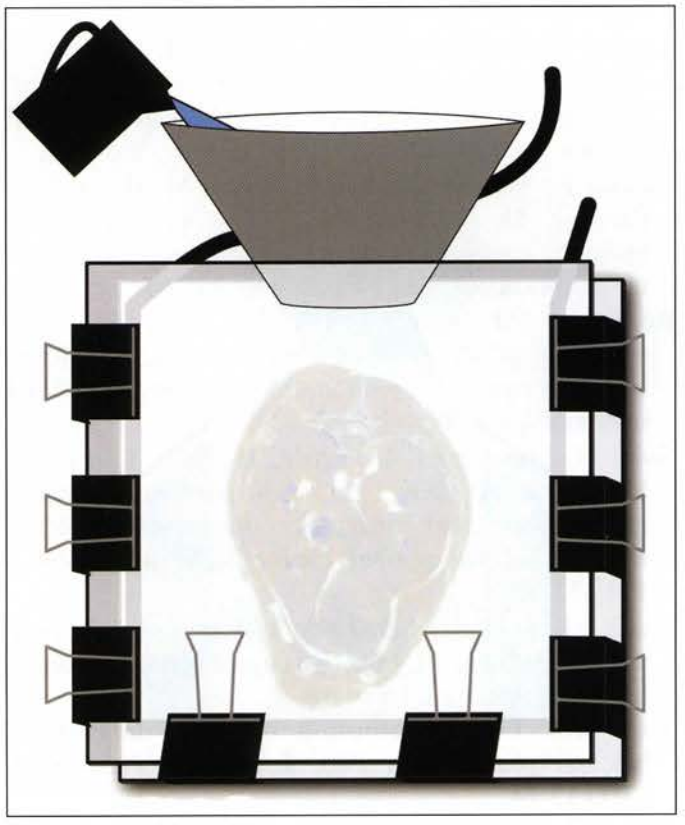

Figure 8. Bottom of fold back clamps turned onto glass. Flat funnel is used to fill chamber with P40 resin.

Figure 9. Closing top gasket and air bubble removal.

Figure 10. Sealed flat chamber ready for horizontal positioning and UVA light curing as the catalyst.

The impregnated slice is surrounded by polyester resin or mixture (P40/A4) while it is curing. Hence, the plastinated slices are incorporated into a single cured sheet of the resin. They are not merely embedded in the resin. The specimens show good delineation of white and gray matter and are durable, as well as demonstrate good anatomical detail for comparison with images of modern imaging modalities (Latorre et al., 2001; 2002; 2004; 2006; Rodriguez et al, 2006; Soler et al., 2007).

Polyester has several advantages over the epoxy technique:

- No time limit to cast the impregnated slices
- Resin used for immersion and impregnation can be reused if no activator is used
- Bubbles rinse faster and easier through the low viscosity resin to the top during casting
- Curing is via UVA-light
- Curing time is shorter and no heat cabinet is necessary
- Plastinated slices do not become yellow

Disadvantages:

- Refractive index is lower
- P40 is light and heat sensitive. Therefore, it is necessary to keep P40 resin in a cold darkened environment as much as possible
- Orange spots may occur in the brain after curing
- Flat chambers usually are sealed for curing

The main advantage of the P40 sheet plastination method is the decreased volume of resin used, two-thirds less, because there is no separate immersion bath; as well, the P40 immersion/impregnation bath may be reused if no activator is used. Also the process is not complicated and less equipment and time is needed. P40 slices are excellent teaching aids for teaching sectional anatomy (Latorre et al., 2001; Henry, 2005c).

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Product distribution:

Biodur Products, Drs. Gunther von Hagens and Angelina Whalley, Rathausstrasse 11, 69126 Heidelberg, Germany. www.biodur.de, Ph: (49) 6221 3311-11, Fax: (49) 6221 3311-12.

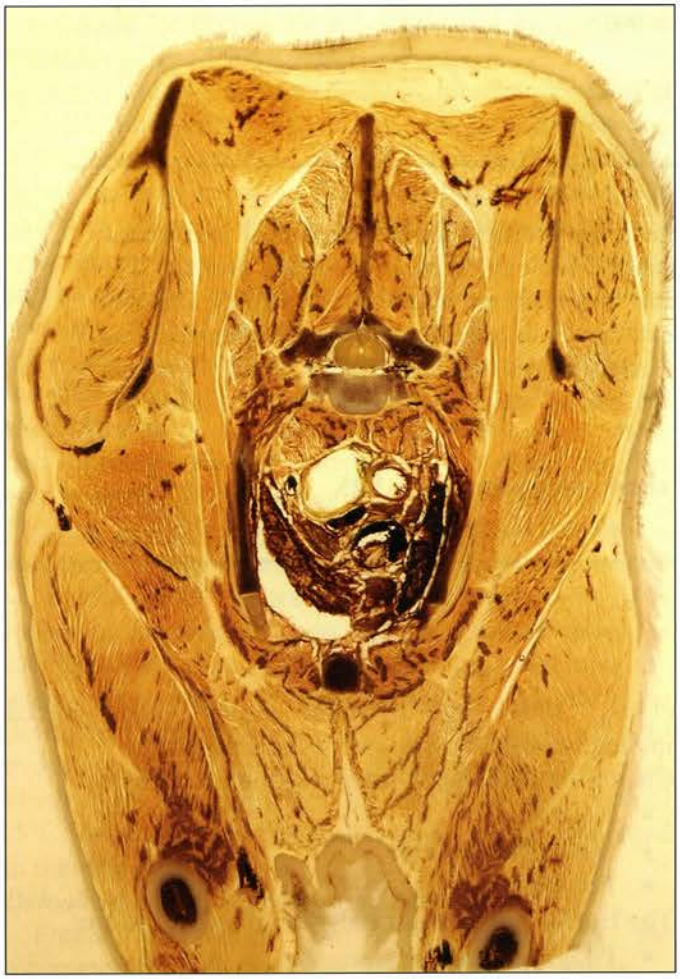

Figure 11. Cured P40 transverse body slice, near thoracic inlet of cat.

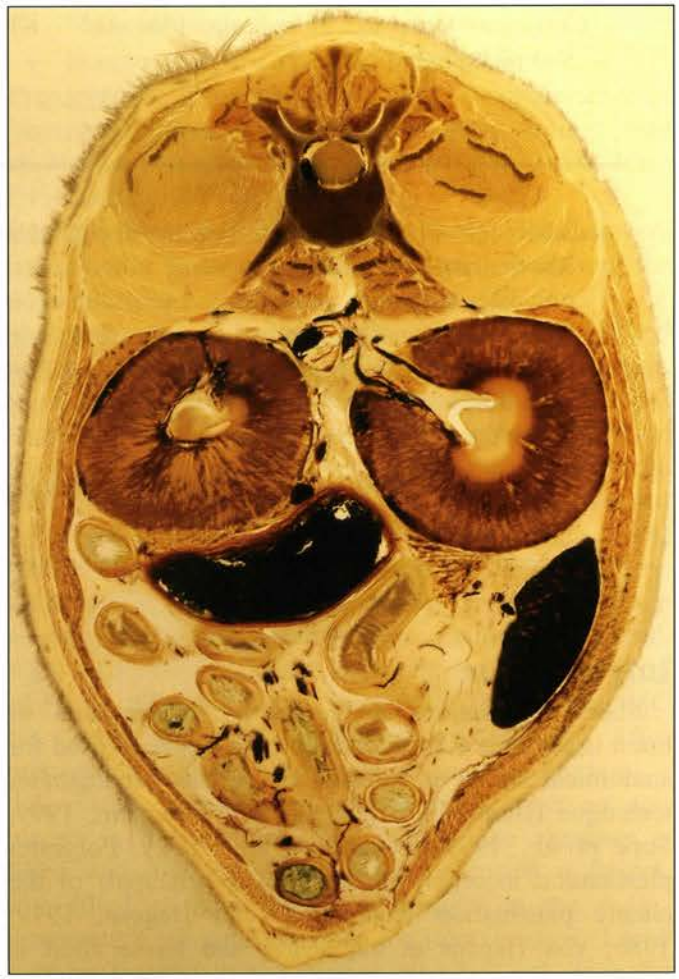

Figure 12. Cured P40 transverse body slice, mid-abdominal cavity of cat.

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Polyester Plastination of Biological Tissue: Hoffen P45 Technique

H.J. SUI[1*] and R.W. HENRY[2]

[1]*Department of Anatomy, Dalian Medical University, Dalian, 116027, China.*
[2]*Department of Comparative Medicine, College of Veterinary Medicine, University of Tennessee, 2407 River Drive, Knoxville, TN, 37996-4543, USA.*

[*]*Correspondence to: Telephone:* (86) 411 - 8480 - 0916; *Fax:* (86) 411 - 8472 - 4558; *E-mail:* suihj@hotmail.com

Abstract: Plastination has become the gold standard for preservation of biological specimens. Plastination is applicable to many allied areas: anatomy, biology, clinical medicine and art. The polyester technique produces 2-3mm semi-transparent to translucent slices which display anatomy within its normal anatomical environs. Polyester slices are an excellent modality for understanding modern diagnostic images: Computed tomography, magnetic resonance and ultra sound. Polyester was developed for preservation of brain tissue. In recent years, polyester is being used for presentation of numerous tissues.

Key words: **plastination; polyester method; body slices; polyester resin; P45**

Introduction

Sheet plastination, developed for preservation of brain tissue in a 4-8mm slice format, has been used for anatomical study or research using the flat chamber technique (Barnett, 1997, Henry and Weiglein, 1999, Sora et al., 1999; Latorre et al., 2002). Polyester plastination incorporates the general principals of the classic plastination techniques (von Hagens, 1979; 1986; von Hagens et al., 1987) and tissue fluid is removed from the slices and replaced with a curable polyester resin. The P35 resin, developed in the late 1980's, is the gold standard for brain slice production of translucent brain slices with exquisite differentiation of white and gray matter. Head slices have been produced with the P35 resin (de Boer-van et al., 1993). P40 brain slices yield good white/gray differentiation. Generally the P40 process utilizes thinner slices (2-3mm) (von Hagens, 1994). More recently, P40 polyester resin is used also for body slices (Latorre et al., 2004) and to plastinate gross anatomical structures (Sora, 1998). A newer polyester (P45) technique will be presented (Gao et al., 2006). All of these resins utilize forced impregnation and casting between glass plates.

Chemicals used in polyester-plastination include:

- Acetone
- Methylene chloride
- Polyester resin

The Hoffen products for polyester plastination are:

- P45: Epoxy resin
- P45A & P45C: Polyester plasticizers
- P45B: Hardener

Materials and methods

The basic steps of plastination are: Specimen prep, dehydration, impregnation and curing.

Specimen preparation

Note for production of P45 body slices, the steps of specimen preparation, slicing and dehydration are similar to the "Biodur™ E12 Epoxy Technique". Please refer to that section of the E12 epoxy process for a more detailed description.

Specimen preparation equipment:

- Bandsaw
- Grids
- Grid/specimen basket

More detail may also be found in the P35 and P40 techniques which precede this manuscript. The non-fixed specimen is positioned for anatomical alignment and frozen preferably in an ultra-cold (-70°C) deep freezer for two days (longer for larger specimens) for best slice production. Fresh tissue may be preferred. However, tissue should be fixed in formalin to decrease the potential for exposure to biohazards (Smith and Holladay, 2001). Tissue color preservation is a prime reason for formalin not to be used. Hairy specimens should be clipped.

Slicing: Large specimens should be divided into smaller manageable portions which will also prevent thawing. Set the guide stop at the desired specimen thickness (2-3mm) and saw serial sections. Cooling the guide stop and saw table prevents premature thawing of the specimen and slices. Square the end of the tissue block and commence sawing. Slices are placed on an acetone resistant grid and the saw dust is removed by scraping it off with a knife and/or running a small-brisk stream of tap water across the surface. Caution: Do not thaw the slice. The grids with their cleaned slices are stacked, tied together with twine and placed in either the first cold acetone (-25°C) bath or in a fixative bath.

Fixation and Bleaching - Optional: Depending on the specimen, it may be necessary to fix the slices, as well as bleach them. Slices can be submerged in 10% formalin for one or two weeks. Once fixation is completed the fixative can be rinsed out in running tap water over night. If brightening of the slices is desirable, immerse them in 5% dioxogen (bleach) overnight or until the desired brightening is completed. Flush with running water for one hour and precool (5°C) to prevent ice crystal formation when submerged in the cold acetone.

Dehydration and degreasing of body slices

Freeze substitution in -25°C acetone is the recommended dehydration procedure for plastination.

Dehydraation equipment:

- Acetonometer
- Specimen/slice basket
- Chemical resistant acetone reservoirs

The precooled, cleaned stack of slices is placed into the first cold (-25°C) acetone bath for one week. Next the stack of slices is placed into the next fresh acetone at -15°C for seven days. The third change is into 100% acetone at room temperature for one week for degreasing. If more transparency of fat is desired, dehydrated slices may be placed into methylene chloride (dichloromethane) (MeCl) for one or two days. Monitor degreasing in MeCl daily. When body slices are appropriately degreased, transfer the slices from their solvent bath (acetone or MeCl) into the impregnation resin.

Forced impregnation of body slices

Forced impregnation, replacement of solvent with a curable resin, is based on a difference of vapor pressure of the solvent and the resin.

Impregnation equipment:

- Vacuum chamber with a transparent lid
- Vacuum pump (oil pump is preferred)
- Vacuum tubing and fine adjustment needle-valves
- Vacuum gauge
- Bennert mercury or digital manometer

Preparing the impregnation-mixture: The polyester resin impregnation bath is made by thoroughly mixing: 1000 ml Hoffen polyester P45 resin with 10g of P45A, 30ml P45B and 5g of P45C.

Immersion into P45 resin of brain slices: Flat chambers will be used to immerse the dehydrated slices into the p45 resin-mix.

Preparing flat (glass) casting chambers for forced impregnation of body slices: Casting chambers are built for casting of the slices prior to impregnation. The flat chambers are constructed of two plates of 5mm tempered glass, 4mm flexible latex tubing and large fold back clamps. The glass and tubing are clamped together around the perimeter of the bottom and sides of the glass. The gasket end, which is left longer, will be used to close the top prior to curing. Once the casts are assembled, the impregnation resin mixture is prepared. 1000 ml Hoffen polyester P45 resin is mixed with 10g of P45A, 30ml P45B and 5g of P45C. P45A and P45C are plasticizers and P45B is a hardener. After preparation of this impregnation reaction-mixture, a dehydrated slice is removed from the acetone and placed in the chamber. Immediately the chamber is filled with the impregnation-mixture using a funnel. The filled chamber is placed upright in the room-temperature vacuum chamber for impregnation. Large bubbles trapped in the casting chamber are manually removed using a 1mm stainless steel wire. The vacuum chamber is sealed and pressure is lowered slowly to 20mm Hg while monitoring for slow bubble release. Similarly pressure is lowered incrementally and slowly through 10mm Hg, 5mm Hg and finally to 0mm Hg while maintaining slow bubble production and release. Pressure is maintained at 0mm Hg until bubbling ceases. Duration of impregnation is eight plus hours.

Heat curing of body slices

After impregnation is complete, the chamber is returned to atmospheric pressure and the chambers

checked for trapped bubbles which are removed with the aid of a wire. Slice alignment is checked and corrected using the stainless steel wire. The gasket is closed across the top and clamped in place in preparation for curing (Table 1).

Curing equipment:

- 40°C water bath and circulation pump.

The slices in their casting chambers, are placed upright in the 40°C water bath for three days. A circulation pump is used to keep water around the chambers the constant 40°C.

Day 0	Prepare and freeze specimen in anatomical position.
Day 1	Slice, rinse, clean and cool slices.
Day 2 or x	Immerse in first cold (-25°C) acetone bath (100%).
Day 9	Immerse in second cold (-15°C) acetone bath (100%).
Day 16	Immerse in room temperature acetone bath.
Day 23a	Build flat chambers; Prepare P45 impregnation-mixture; Insert dehydrated slice into flat chamber and fill with resin-mix.
Day 23b	Insert filled flat-chamber into vacuum chamber; Impregnate with P45.
Day 23c	Remove chambers after impregnation; Inspect for bubbles and alignment.
Day 23d	Cure - Place in 40°C water bath.
Day 26	Remove from water bath and cool.
Day 27	Open flat chamber, cover slice with foil, saw and sand

Table 1: General protocol for P45 - polyester body slices (2-3mm).

Finishing: After curing, the sheets in their flat chambers are removed from the water bath and cooled to room temperature. The flat chamber is dismantled by removing the clamps, gasket and glass. The specimen is wrapped in light weight foil to prevent any uncured resin and debris from contacting the surface of the slice.

After curing, release and wrapping is complete, the excess cured resin is trimmed on a band saw. The edges may be smoothed using a wool sander and new foil is placed on the slice which is ready for use.

Results

The P45 sections are semi-transparent (Fig. 1), durable, and correlate well with radiographic, CT and MR images.

Discussion

The dehydrated slices placed in the open topped flat chambers for impregnation is a potential time saver. As well, heat curing in a water bath in the same chamber after closure of the top is unique and a time saver. As with the other polyester techniques, the impregnated slice is surrounded by polyester resin-mixture (P45) while it is curing. Hence, the plastinated slices are incorporated as a part of a single cured sheet of the resin. They are not merely embedded in the resin. The specimens in the slice are durable and show good anatomical detail.

The main advantage of the P45 sheet plastination method is the decreased volume of resin used. As with P40 the impregnation resin is used as the casting resin. The process is not complicated and less equipment and time is needed.

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Product distribution:

Dalian Hoffen Bio-technique Co. Ltd, Room 301, No. 32, Lixian Street, Hi-Tech Zone, Dalian, China. URL: http://www.hoffen.com.cn; Fax: (86) 411-8475-4558 e-mail:wangjian599@hotmail.com

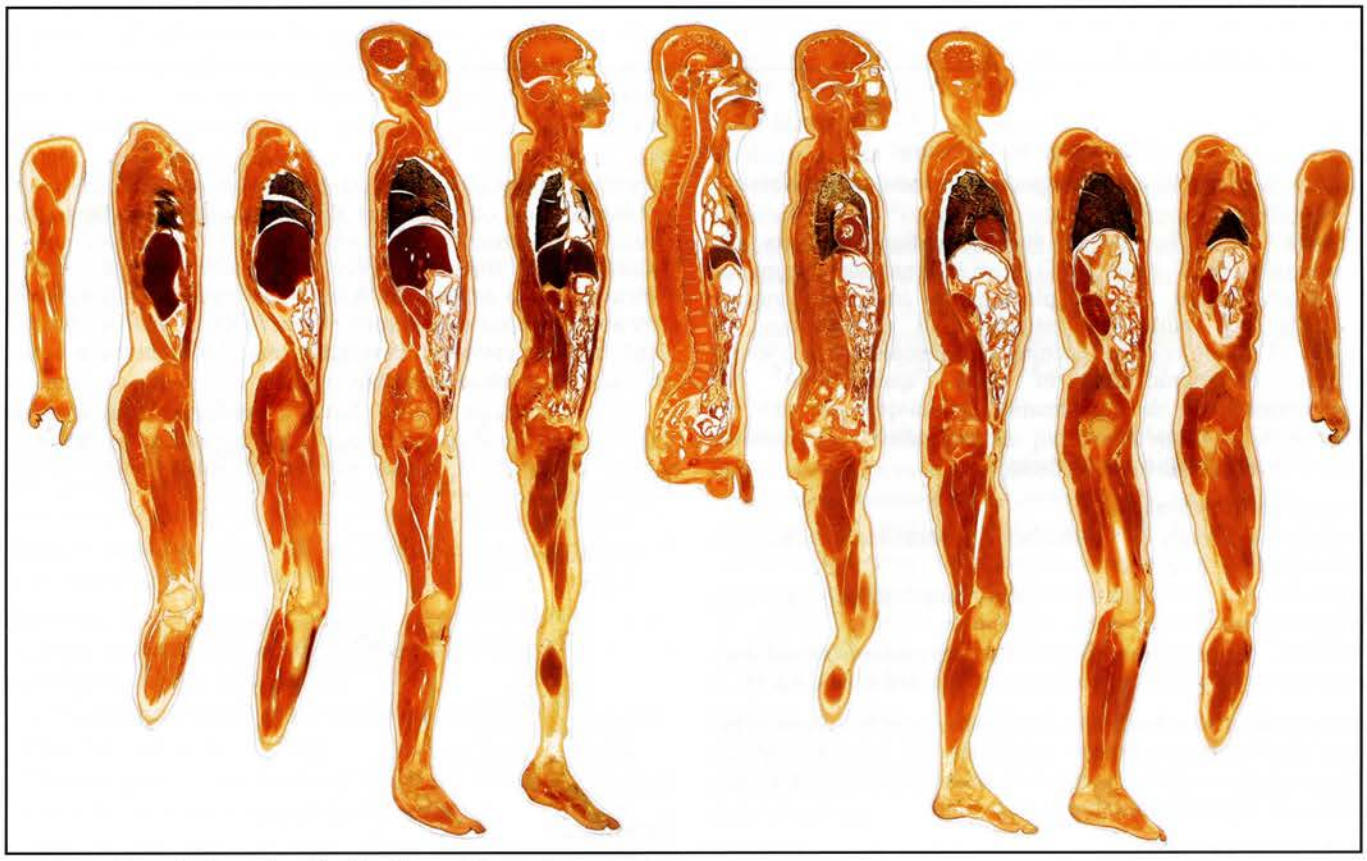

Figure 1. Cured p45 sagittal body slices of human.