Chapter 9
Frozen Section Technique in the Animal Research Setting

Charles W. Scouten

Abstract There are significant differences between the skills required for research animal and for clinical histology. Researchers normally use sacrifice perfusion to harvest animal tissue, and need to freeze and section whole organs, sometimes in a specific orientation. Clinical histologists normally work with small samples or fragments, immersion fix, and paraffin embed.

Perfusion clears the tissue of red blood cells, which interfere with many common cell labeling methods and block capillaries to the entrance of fixative. High perfusion pressure clears red blood cells faster and more thoroughly, and achieves faster fixation. Pre-wash must be isotonic, preferably sucrose, while the fixative solution should be hypotonic, because the cytosol becomes hypotonic during pre-wash, to avoid shrinkage of soft tissue.

Tissue that is to be frozen in order to harden for sectioning must be snap frozen throughout, a greater challenge when large blocks of tissue such as whole organs are used. Liquid nitrogen will freeze faster and create a shell around the exterior of the tissue, and then the organ will crack when the interior expands due to slower freezing. Freezing tissue in a slurry of dry ice and isopentane works better for rodent brains or similar size blocks of tissue. In setting up the microtome, blade angle should be adjusted to equal the bevel on the lower edge of the knife, regardless of the method of hardening or sectioning, and regardless of the tissue being sectioned. Commercial gelatin encasement (Brain Blockers™) can provide accurate, reproducible orientation for rodent brains. Tape transfer methods provide accurate transfer from the frozen block to the slide, with all fragments in original orientation and relative position.

Keywords Perfusion • Freezing artifact • Snap freezing • Organ orientation • Fixation • Organ shrinkage • Sectioning • Brain blockers • Peltier freezing stage • Tape transfer system • Cryostat • Sliding microtome • Isopentane • Liquid nitrogen • Gelatin encasements • Blade angle • Swiss Cheese Artifact

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9.1 Introduction

Oddly enough, histology on research animal tissue is generally quite different from histology on human biopsy or post mortem tissue. Both are mammalian tissue, and to be sectioned for observation under a microscope. However, from the beginning, animal researchers gain the advantage of rapid and reproducible fixation and thus, better quality tissue preparations by employing sacrifice perfusion. In most cases, tissue must be hardened for sectioning. To harden, animal tissue is much more likely to be frozen as whole organs rather than paraffin embedded as pieces, or frozen as small pieces. Animal research sometimes requires tissue to be cut without hardening by employing a vibrating microtome. For animal research work, brain and other tissues are usually required to be sectioned accurately in specific orientation. The use of perfusion fixation rather than immersion, sectioning of whole organs, and freezing or vibrating microtome rather than paraffin sectioning is characteristic of most research animal tissue histology, but are rarely encountered in the clinical lab. The end result is a frequently different skill set required for histology on research animal tissue vs. clinical tissue.

The author's primary experience is in the area of brain research and so will discuss issues relating to frozen section in animal tissues in the context of brain tissues.

9.2 Sacrifice Perfusion

Animal researchers commonly sacrifice an animal from which internal tissue samples will be harvested by the following method:

1. Heavily anesthetize the animal
2. Open the chest cavity
3. Cut a knick in the wall of the left ventricle of the heart
4. Insert a gavage needle into that ventricle
5. Flow an isotonic pre-wash solution through the circulatory system
6. Flow a fixative through the circulatory system

The cardiovascular system provides an open parallel channel to every live cell in the body in seconds. Washing out the blood through a needle in the heart, and then running fixative through the vascular channels, enables very rapid fixation of every part of the whole body (Palay et al. 1962; Scouten et al. 2006; Garman 1990).

Living cells have active, energy-using ion pumps on the cell membranes. Any challenge in the cellular environment inside a warm blooded mammal, whether thermal, pressure, chemical, or anoxia, shuts down these pumps. Changes in cellular contents begin almost immediately as ion distributions change. Cells begin a process of autolysis (self digestion) almost immediately at the onset of anoxia, with the ultrastructure breaking down before immersion fixation could occur (Palay et al. 1962; Cammermeyer 1960). Thus, from first breaking into the

chest cavity, the time until arrival of fixative should be minimized to achieve reproducible tissue quality by beginning fixation as close as possible to the living state.

There are other advantages of perfusion. Red blood cells catalyze the reduction reaction of DAB (3,3′ Diamino Benzidine Tetrahydrochloride) even better than Horseradish Peroxidase (HRP) does. Red blood cells also autofluoresce, sometimes obscuring or creating background staining in cases where a florescent label or stain has been used. Red blood cells occasionally block a capillary in living tissue at resting blood pressure, and eventually break free. Thus, any red blood cells remaining in the tissue after perfusion probably blocked fixative from reaching some areas as rapidly as otherwise possible at best, and at worst, it may interfere with visualizing a label or stain commonly used by animal researchers and meant to mark certain cells, cellular ultrastructure, or specific sites in the tissue.

In contrast, biopsy-obtained or other human tissue must be immersion fixed with the blood still in it. Formaldehyde penetrates intact tissue slowly (about 18 mm in 25 h; diffusion = $3.6 \times \sqrt{\text{time}}$) (Medawar 1941; Baker 1958), so human tissue must be cut in small pieces, with all parts of the volume within a millimeter of an exposed surface, to get reasonably uniform fixation and tissue quality throughout a given volume of tissue. Scientists working with research animal tissue usually prefer to section whole organs, such as whole brain, to see and interpret the track of a probe insertion, or to determine the anatomical location where cells were stained. Both paraffin embedding and immersion fixation requires the tissue to be small or at least thin pieces. Thus, the need to section whole organs is another reason why animal tissue is most commonly both perfused to fix throughout, and frozen to harden for sectioning.

A drawback of the perfusion method is that perfused soft tissue is commonly shrunken by the process. Under an electron microscope, the perfused brain is usually seen to be devoid of extracellular space (Van Harreveld 1972; Cragg 1980), while other lines of evidence show that the living brain has about 20% extracellular space (Van Harreveld and Steiner 1970; Van Harreveld 1972). Correspondingly, the brain is shrunken in whole organ size by as much as 20%. In some cases, this is not a problem for the purposes of the project, but shrinkage and anatomical distortion is frequently a drawback with neural tissue, where the purpose of histology is to define a location in the brain.

Shrinkage and resulting distortion was and are accepted by most scientists as an unavoidable consequence of tissue processing, and is described as such in a stereotaxic atlases of the brain "*This method needs some comment. It inevitably implies shrinkage caused by embedding and staining. Shrinkage cannot be equalized by enlargement because, for physical reasons, the extent of shrinkage differs in the various constituents of the brain*" (König and Klippel 1967). Later sections in König and Klipple made it clear the formaldehyde was the part of "embedding and staining" that caused the shrinkage. As a result, König and Klippel (1967), could not provide accurate stereotaxic coordinates that can be applied to living brain. Paxinos and Watson (1998) avoided this problem by working only with fresh frozen tissue, and not fixing. Of course, many histological reactions do not work with fresh, unfixed tissue.

Although perfusion is ubiquitous throughout animal research work, the procedure varies between laboratories, and little is ever said in the methods section about what procedure was used. Until recently, there has been no commercial instrument for perfusion. Each lab cobbles together its own apparatus for this job. This is unfortunate, because how it is done affects tissue quality and reproducibility of immunological and other stains.

The first and a still common method is to use gravity to drive the flow (Palay et al. 1962). Two bottles with tubing nipples at the bottom are placed on a shelf above the drain surface where the animal will be placed. Tubing from each bottle is connected to the arms of a "Y" connector to form a final common flow line, with clamps on the outlet from the bottles. The experimenter first flows the pre-wash for a short period whose length is sometimes determined by volume passed, sometimes a few minutes, and then flows the fixative. Typically there is not even this much detail in the methods section. Tissue so perfused is seen to have a reddish hue, and has red blood cells throughout. This means that some capillaries did not clear of red blood cells, and so must not have gotten fixative flow. The reason can be calculated. Even in living animals, capillaries stop up, with a cell stuck for a while, then clear. Even normal blood pressure does not always push blood cells along on every branch, with every beat. Clearly, at least physiological pressure should be used in perfusion. A pressure higher than maximum systolic pressure would be needed to force out all red blood cells. So the water bottles should be positioned to provide a pressure of at least 150 mmHg, a high systolic pressure most mammals can reach when exercised. One mmHg equals 13.6 mm H_2O. Thus, to achieve even physiological pressure, the source bottles should be over 2 m above the animal, even if no pressure is lost in the needle. Given ceiling heights above the countertops, this is not possible in the great majority of labs. This fact accounts for the generally poor perfusions seen, with blood remaining in the tissue.

A more recent practice is to use a peristaltic pump to drive perfusion fluid. However, this controls flow rate, not pressure. There are no algorithms to calculate the correct flow rate to create sufficient pressure, to clear every capillary in a given animal. Experimenters develop their own flow rate empirically, finding what works in the animals they commonly use, and typically can get a flow rate that yields better results than gravity. However, the vascular resistance, and so the correct flow rate, will vary with growth, age, species, strain, gender, exercise, and health state to name a few. The same flow rate used on a young and an old rat will get different results, and create a systematic bias in fixation and tissue quality, and at least in immunological reactions. Degree of fixation affects antigen binding, enzyme activity, and other relevant functions. If the flow rate chosen creates sufficient perfusion pressure for small rats, larger rats will be less fully perfused and more slowly fixed, and perhaps have more background noise in the stain. Consider perfusing a mouse and a rat at the same flow rate. Whatever rate is chosen, one animal will yield suboptimal results. However, within limits, all mammals have the same blood pressure, and a selected pressure can be used in any mammal, even neonates, and give consistent, reproducible results. A pressure of 170 mmHg would be an above resting pressure in any mammal. See Table 9.1.

Table 9.1 Average blood pressure for several species (systolic/diastolic) (Green 1979; Short 1987)

Animal	Average blood pressure
Mice	113/81
Rats	116/90
Hamster	150/110
Rabbit	110/80
Dog	112/56
Cat	120/75
Baboon	148/100
Rhesus monkey	160/127
Pig	170/108

Table compiled from both sources
Mice 113/81 Rats 116/90 Hamster 150/110 Rabbit 110/80 Dog 112/56 Cat 120/75 Baboon 148/100 Rhesus 160/127 Pig 170/108

Therefore, it is easier to select a pressure that is appropriate across species, genders, strains, weights and individuals than it is to select a flow rate for each individual.

Clearly, it makes more sense to control pressure, but it must be much more pressure than gravity flow from bottles the experimenter can reach by hand.

Many experimenters say that they adjust pressure or flow rate down if they see clear fluid coming from the nose. Rodents have a pressure release valve that allows excess cerebrospinal fluid (CSF) to pass through the cribiform plate and out the nose (Brodbelt and Stoodley 2007). Perfusion is putting excess fluid into the system. Of course it will activate the overflow system if applied under even physiological pressure with a continuous inflow. If clear fluid is coming out the nose, it is proof that the CSF and the extracellular fluid are being replaced by pre-wash or fixative, as you should hope. There is no reason to back off the pressure on account of this flow. If red blood were to come out the nose at the beginning of perfusion, that would indicate vascular rupture, and would be a problem. Clear fluid does not indicate vascular rupture.

9.3 Avoiding Shrinkage

Cragg (1980) devised a complex apparatus to maintain above physiological pressure, 300 mmHg, and found he could get brain tissue for electron microscopy in which the extracellular space was retained. He did not take a measure of whole organ size. There was no noticeable damage to tissue, even at the EM level, caused by the short-term application of pressure even that high above physiological pressure.

A commercial apparatus for sacrifice perfusion has recently been introduced by Leica's MyNeuroLab.com (St. Louis) subsidiary (*see* Fig. 9.1). The project to develop and test this was supported by Small Business Innovative Research (SBIR) grant funds from National Institutes of Health/National Institute of General Medicine (NIH/NIGM).

Fig. 9.1 Perfusion Two™ apparatus for sacrifice perfusion. The bottle on the left serves as an air pressure tank, the next bottle is prewash solution, then fixative. The 4th bottle is optional for a postwash if desired

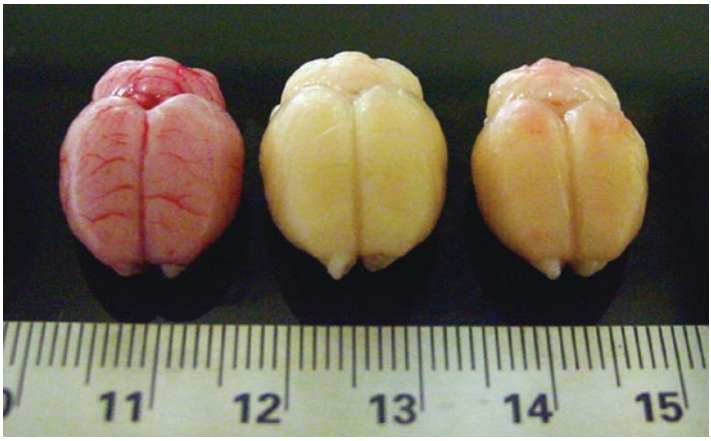

Fig. 9.2 Perfusion Comparison. Three brains perfused by different methods. The brain on the left is fresh, unperfused tissue. The middle brain was perfused at 300 mmHg, and the brain on the left was perfused by gravity pressure in a typical setup. Tissue preparation and photograph by Dr. Miles Cunningham, McLean Hospital

The Perfusion One™ and an automated version, the Perfusion Two™, control pressure up to 300 mmHg, and come with a protocol for reproducible perfusions of reliable consistent quality. The 300 mmHg pressure (over 4 m of water) fully clears nearly all red blood cells see Fig. 9.2. The Perfusion One also provides a fourth

perfusion fluid bottle for post wash. Immunological reactions require that the tissue be fixed, but not for too long. The animal can be perfused; left with fixative inside or on slow flow for 4–6 h, then the fixative washed out, perhaps with 30% sucrose or other cryoprotectant solution, and be fully saturated with cryoprotectant and ready to section immediately after. The tonicity of the perfusion fluids, and the pressure used, can enable a perfusion without shrinkage (Scouten et al. 2006).

9.4 Tonicity of Perfusion Fluids

Osmolarity is a measure of the number of particles (ions or molecules) in a given volume of water. Tonicity refers to distribution of particles on either side of a semi-permeable membrane. Only particles that cannot cross the membrane contribute to tonicity. For example, the inside of cells is typically about 330 mM in osmolarity. If fluid outside the cell has a different osmolarity, either water or particles or both will move to balance tonicity. Particles will move down any concentration gradient, if they can cross the membrane, like sodium leaking into a cell. Urea moves freely through a cell membrane, so the osmolarity inside and outside the cell would rise with urea concentration, but this would have no effect on tonicity, and thus no effect on water flow. Likewise, a 4% formaldehyde solution, even without buffer, is about 1,400 mM, but formaldehyde is believed to freely cross the cell membrane (Cragg 1980; Thrift 1997; Maser et al. 1967), and so has only transient, if any, effect on tonicity, and thus no direct tonicity effect on movement of water across a cell membrane. Formaldehyde does stop energy use, stops all ion pumps, and changes permeability of the membrane, and so has significant indirect effects on tonicity.

Sucrose at 9.25% concentration, or sodium chloride at 0.9% concentration, are isotonic solutions, that is, they will not cause significant volumes of water to move in or out of a living cell bathed in them. However, sodium is constantly leaking into cells, and being actively pumped out of cells with an energy-using metabolic process, to maintain a roughly 10:1 ratio of sodium outside to inside the cell. If a cell is challenged and its energy mechanisms slowed or stopped, as in the anoxia and thermal shock of perfusion or the arrival of fixative, sodium will flow in and not be pumped out. The cell will swell as it takes in water from the extracellular space, down the concentration gradient, to maintain tonicity by diluting the inflow of sodium. It will take water from the extracellular space and move it into the intracellular space. Possibly this accounts for the loss of extracellular space in perfused tissue. Although the water and excess sodium move out when fixation has made the membrane more permeable and particles rebalance, the extracellular space appears not to reopen. Instead, the fluid moves out the vascular system and the whole organ shrinks. Sucrose, in contrast, cannot cross the cell membrane.

A *hypertonic* perfusion fluid would shrivel the tissue by drawing water out of cells and out of extracellular space, and into the vascular space and hence out of the animal. Use of *hypotonic* perfusion solution would cause cells to take on fluid (from the vascular space, through the extracellular space) and swell. Cellular swelling

is commonly reported as challenged cells start to fail metabolically. This is, in part, due to the influx of sodium when the pumps shut off and water flowing in to balance tonicity, and may be partly because autolysis, internal breakdown, expands the number of particles in solution and increases internal tonicity. If swelling eliminates the extracellular spaces, and is transient, the subsequent outflow of fluid to the vascular space would result in shrinkage of the whole organ. This author (unpublished) has seen shrinkage of whole organ in mouse brains in which a 5% sucrose solution was used for the pre-wash, and the fixative solution was isotonic. Thus, hypotonic or hypertonic pre-wash may have a final result of shrinking the brain, given an isotonic fixative solution.

Cragg (1980) reasoned that the loss of extracellular space (and whole organ shrinkage?) could be prevented by removing sodium from the extracellular space before fixative arrived, or swelling irreversibly emptied and closed it. He used 10% sucrose (~isotonic) as the pre-wash solution, rather than the conventional saline or phosphate buffered saline (PBS). This would be sufficient to wash sodium out of the extracellular space for most tissues, but due to the blood brain barrier, sucrose does not leave the vascular compartment in the brain. To force it out, and thereby replace the extracellular fluid, he applied a pressure of 300 mmHg, shown to force sucrose across the blood brain barrier (Rappoport 1976). Again, this resulted in no detectable damage caused to the tissue, even under the electron microscope. He was able to balance tonicities with a complex mixture and prevent the loss of extracellular space (and prevent whole organ shrinkage?). Sucrose cannot cross the cell membrane, and therefore does not move down its concentration gradient into cells, and so contributes strongly to tonicity.

Perfusion is complex dynamically. Opposing processes are occurring. The pre-wash solution of isotonic sucrose should be carrying away any ions that can cross the cell membrane, including most of the sodium, as they move down the concentration gradient into the passing fluid. This should act to prevent or delay the early cellular swelling. Conversely, autolysis (cell internal breakdown) would be increasing the particle count inside the cell, which would tend to increase fluid inflow and cellular swelling. Unless these new breakdown particles can leave the cell down a concentration gradient and be carried away, the balance between these two processes in time would determine if swelling will occur.

The steady flow of sucrose through the vascular and extracellular space would remove any dissolved particle that can cross the cell membrane, thus the internal tonicity of the cell should drop as the pre-wash proceeds. Water would then also leave the cell, maintaining the extracellular space. Formaldehyde in water, no saline or buffer, following a pressure isotonic sucrose pre-wash, has been seen to swell the organ size above normal, as measured with a plethysmometer immediately post perfusion (Scouten and Cunningham, unpublished). Thus, the size change seems to be purely a tonicity issue if the extracellular space has not been closed by initial swelling. If shrinkage occurs, it is then due to isotonic fluid on the outside, hypotonic fluid on the inside, by the time the fixative arrives. Water leaves the cell via the vascular space rather than reopen the extracellular space. The cell had been previously swollen, so that the cell had displaced the extracellular space, which does not reopen, and therefore the whole organ shrinks as the water leaves.

In summary, we recommend a pre-wash of isotonic or 10% sucrose forced through at 300 mmHg, and hypotonic fixative in about 3% sucrose, to avoid shrinkage or expansion. The exact numbers have not been fully resolved. However, these solutions will give better results than conventional isotonic solutions. The sucrose will cause the muscles and limbs to move slowly for a few seconds until the sodium is gone. When the movement stops, it is a good end point to open the fixative clamp and let it flow. Let it mix with sucrose for a while, about ten seconds, then close the sucrose pre-wash clamp. Let the pressure remain at 300 mmHg until the fixative reaches the animal, and this will depend on the internal volume of the tubing to the animal, and the flow rate through the animal. When some stiffening of the animal's muscles is noted, you can drop the pressure to about 100 mmHg to avoid fluid waste. Fixative does not need to flow fast, just keep the mix inside refreshed, once the blood is already out.

The tubing internal volume, the diameter times the length from the animal back to the "Y" connector, is critical. Too thin a diameter and the pressure drop due to resistance in the tubing will use up the pressure and not force the blood brain barrier, and may not force out all the blood. Too large, and the volume of fluid in the tubing will take too long to clear the animal, and thus needlessly delay the arrival of fixative after the switch. A tube appropriate for the rat would take an excessively long time to switch fluid to the mouse, given the much slower flow rate. The animal cardiovascular resistance must be determining the flow rate. A test would be appropriate to time how long it takes to empty a bottle with or without the animal, but with everything else (tubing to the needle used). It should take much longer with an animal involved. If not, the needle or the tubing is the bottleneck, and needs to be larger. The thinnest tube that meets these criteria is ideal.

9.5 Tissue Orientation

For many tissues, how an organ is oriented for sectioning is not important. For others it is. Frequently, skin or retina may need to be cut in cross section. To cut thin tissue pieces along an edge, sandwich the tissue between two strips of balsa wood that has been soaked in media, and then clamp or freeze the wood, tissue and all, and section the entire assembly. Balsa will cut easily.

Brain nearly always needs to be cut in a specified plane, usually the plane used in atlases, coronal (defined perpendicular to skull flat), sagittal and horizontal. This may be accomplished, crudely, by taking a razor blade and cutting a flat plane through the brain approximately perpendicular to cortex. Then put the cut surface of the needed block of tissue on a pedestal, and freeze for sectioning. The cryostat or microtome should have an adjustable pedestal to tilt the tissue. After a few sections, the user notes the orientation error, adjusts some, and tries again. Trial and error adjustment by observation before reaching the critical region from which sections are needed is a commonly used procedure, and is time consuming and error prone.

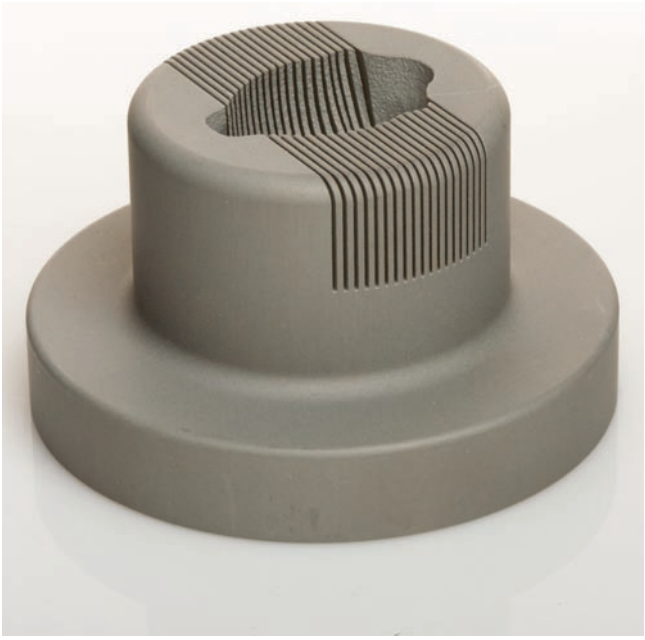

Fig. 9.3 Rat brain coronal matrix. Note the approximately brain shaped cavity

Several manufacturers make matrices, like a carpenter's miter box with slots positioned a mm apart, and perpendicular to a floor shaped like the curvature of the brain surface. This improves the accuracy of the initial cut-through considerably over the free-hand operation, see Fig. 9.3.

These matrices may be made of metal or acrylic. However, matrices are not a final solution. Like cutting cheese with a knife, the tissue sticks to the blade as it passes through, and the chamber fits loosely, there is room for the brain to move or be somewhat out of position. Thus, the cuts are not fully reproducible, even if the same slot is used each time. Some trial and error adjustment is still needed. This can be improved by using a surgical monofilament to make the cut through, instead of a blade, again like a cheese slicer. It may be necessary to cut the membranes at the brain surface with a blade and then use the monofilament.

A new solution has recently been offered. Gelatin encasement blocks (Brain Blocker One™, Leica Biosystems, St. Louis) are cast from a mold, originally cast from the fixed brain (without shrinkage) of an adult rat or an adult mouse. These gelatin blocks have rectangular sides, and the brain cavity inside is oriented in the atlas planes relative to the sides. The block can be put on a flat pedestal and sectioned, gelatin and all, after a brain is encased inside. Figure 9.4 shows a fresh brain being encased.

These sections should be highly reproducible, and always in the atlas plane in which the blocks were originally cast. Soaking the gels in media or water slowly

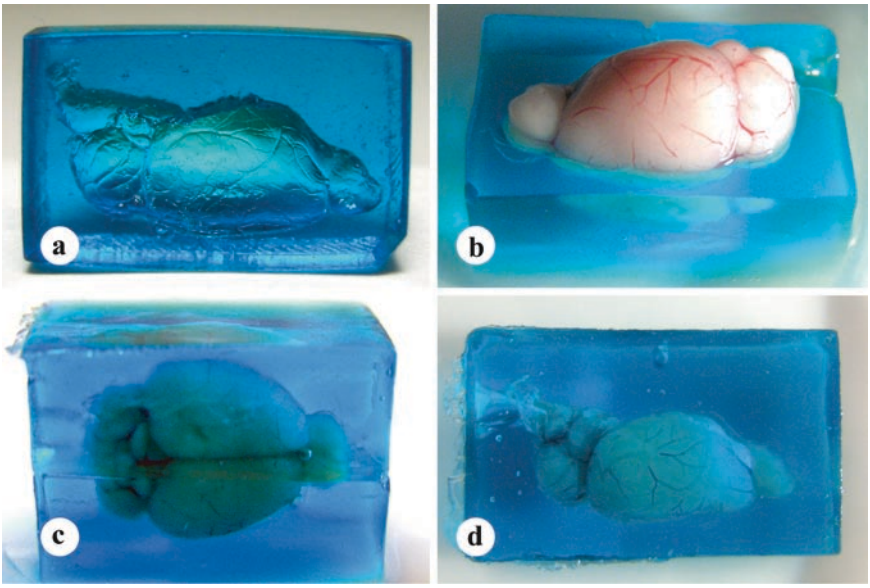

Fig. 9.4 Mouse brain being encased in Brain Blocker One™ (**a**) Empty mouse brain cavity in stereotaxic alignment in a half gelatin block (**b**) Mouse brain, fresh tissue just extracted, settled in one half of the gelatin block (**c**) Mouse brain completely encased in gelatin block. Seam just visible. (**d**) Placed on back, edge aligned parallel to skull flat, ready to freeze and section gelatin and brain together

expands them, so that size differences due to age or strain of a group can be adjusted by soak time.

The Brain Blockers™ can be used one at a time to improve orientation, and thus save time with trial and error adjusting on the cryostat, or the rectangular blocks can be placed side to side against each other, and sectioned all at once in each pass of the knife. Mouse brain sections made in this way can be placed on 1 × 3 slides (up to 12) or 2 × 3 slides (up to 24). If large sheets of several brain sections are made, it may be necessary to collect them with a tape transfer system (CryoJane™ or Macro CryoJane, Leica MicroBiosystems, St. Louis). See below. Blocks of 24 also require greater blade movement range than is common for research laboratory cryostats, so a large format cryostat with greater movement range may be needed (Model 8850, Leica Biosystems, St. Louis). Note that in so doing, you can section once through the critical area on 24 brains at once, and enjoy a nearly 24:1 reduction in time needed to complete sectioning of all brains in an experiment. Further, consider the resulting slide layout. The same region from each brain is on one slide, and all are sectioned in the same atlas plane. Comparison should be greatly facilitated and the microscope analysis time correspondingly reduced.

9.6 Tape Transfer Technique

When large slices of tissue, or multiple small pieces that should be kept together in order are to be sectioned, or when the collected sections must be perfect for pictures, as in an atlas (Watson et al. 2009), a tape transfer instrument is very effective (CryoJane Tape Transfer System™, Leica Biosystems, St Louis). The concept is simple. A specially coated piece of tape is applied over the cut surface of the block with a roller. When the next section is cut, the blade will pass through the tissue below the tape. The tape will adhere to the section exactly as it was on the block. The tape is then placed on a specially coated slide, and given a brief exposure to UV light. The UV light releases the adhesive on the tape, and activates an adhesive on the slide. Simply pull the tape off, and the section remains on the slide, exactly as it was on the block, without stretching or distortion, and with anatomic structures in position and orientation, even if that plane of section has disconnected parts. Figure 9.5 shows the CryoJane parts to be installed, some inside and some next to the cryostat.

9.6.1 Choice of Sectioning Method

Animal researches usually want to section whole organs or large sections. Paraffin penetration would typically be too slow for whole brain sectioning, for example. Commonly, researchers section either frozen tissue with a cryostat (Fig. 9.6), or sliding microtome (Fig. 9.7) with freezing stage, or soft tissue with a vibrating microtome. There are issues to be addressed with either and both methods.

Fig. 9.5 CyroJane™ Components

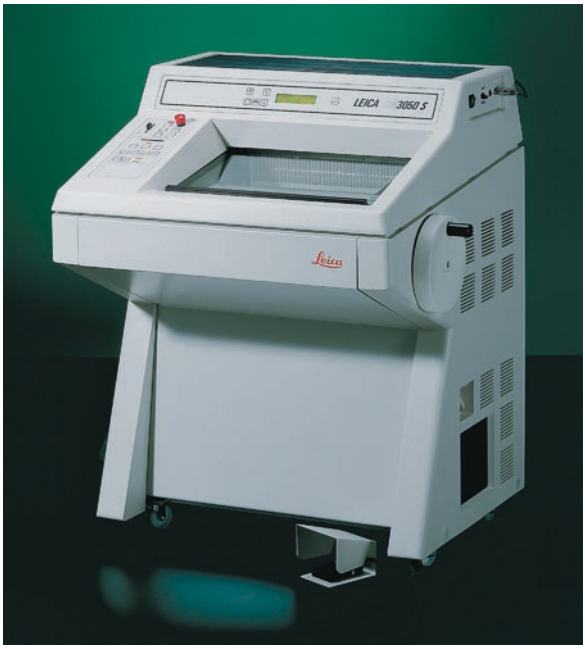

Fig. 9.6 Cryostat for frozen sectioning

Fig. 9.7 Sliding microtome for sectioning tissue

9.7 Blade Angle

Blade angle is an issue about which there is a lot of unnecessary confusion and superstitious behavior for both vibrating and frozen microtomy. Incorrect blade angle is a frequent cause of problems with sectioning tissue. The blade moves forward in a given plane. The blade body is usually tilted at some angle to that plane. This angle between the plane of motion and the blade sides is the blade angle, as frequently referred to on histology help pages. In Fig. 9.8, the angle between the direction of motion and line C–D is the blade angle.

Blades have a common feature in the shape, a final bevel of about a millimeter to the edge, see Fig. 9.8. The bevel angle is usually unknown to the user, but is critical information that should be measured or obtained as closely as possible. Manufactures should laser this information on disposable blades intended for use in histology. At present, the user can obtain a common protractor, place the blade on a glass or other flat surface, and push the edge down so the bevel is flat on the surface. Measure this angle with the glass to the body line of the blade (line C–D).

If the blade shown advances straight to the right into the tissue block, the edge at B will cut a thin section off the tissue block. If the blade were more horizontal (point D lowered some), then the edge from B to C, which is the lower bevel, would forcibly compress the tissue behind the cutting edge. Too low a blade angle leads to a common problem, alternating thick and thin tissue sections (this can also be caused if anything is loose and can move even slightly, such as the blade holder or the pedestal the tissue is on, or if the tissue is flexing away from the blade, as when a vibrating blade is advancing too rapidly). The softer the tissue, the more it will be crushed and compressed by too low a blade angle. Hard tissue may force the blade to deflect upward, and leave the block and skate along the top of the block.

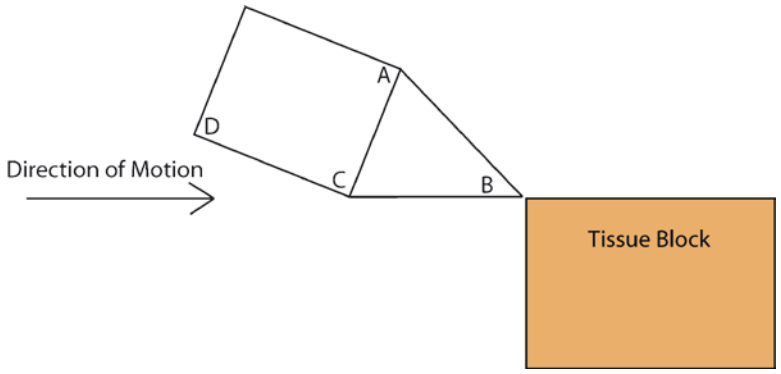

Fig. 9.8 Diagram depicting blade bevel and blade angle. ∠ABC is the total bevel angle. This is sometimes asymmetrical relative to the midline, the bevel may be only on one side. Lower bevel angle is the angle of line BC relative to the midline of the knife body. Plane BC is the lower bevel face. Line BC should be parallel to the direction of motion, except with C slightly higher. Line CD's angle to the direction of motion is the Blade Angle

As tissue is cut, the blade bevel forces the tissue to bend sharply up at the cutting edge of the blade. The upper surface of the section above the blade is compressed. The lower surface in contact with the blade is stretched. The steeper the blade angle is set, the more sharply the tissue is forced to bend. The consequences of too much bend are worse if the tissue is very hard, and it may break (shatter marks parallel to the blade), curl or deform. A steep blade angle forcing the tissue to bend will be more damaging to hard tissue (may shatter) than to very soft, flexible tissue, which can bend easily. Probably because the consequences of incorrect blade angle vary with direction of error, and with tissue type and hardness, and blades vary in bevel angle from manufacturer to manufacturer, many histologists believe that the correct blade angle depends on the tissue being cut, and must be determined by trial and error. It does not. It is never advantageous in histology to have the bevel trailing a flat surface compressing the tissue behind the edge (too low a blade angle). And it is never advantageous in histology to bend the section more sharply than necessary at the cutting edge. This leads to the conclusion that the correct blade angle, whether on a vibrating microtome or a cryostat, is always the blade's lower bevel angle. Modify this very slightly. So that the bevel face does not slide over the freshly cut surface with friction abrasion, the blade angle should be above the bevel angle, but as little as possible. A compromise position is unlikely to ever be warranted because the consequences of too low a blade angle are more damaging than the slight increase in bend angle needed to rectify.

Blades with high bevel angles are stronger and last longer. Blades with low bevel angles bend the tissue less and should slice into tissue easier, with less compressive resistance yielding flatter sections, but the edge is more fragile and less durable. Of course, sharpness of the blade is a separate and very important issue as well, as is hardness of the blade material see Chap. 4 page.

9.8 Vibrating Microtome

The advantage and purpose of a vibrating microtome is that it can section a block of soft tissue under liquid, without previously hardening the tissue. The processes of freezing tissue and paraffin embedding tissue are alternate means of sufficiently hardening tissue so that a knife will cut cleanly through the tissue, rather than compress the tissue in front of the blade. Both of these techniques can have significant drawbacks for animal research. Freezing commonly fractures some cell membranes (but see below), and lets cell contents in the cytosol leak out. Thus, vibrating microtome-cut tissue usually has better preservation of cell contents, and labeling stains such as HRP or fluorescent compounds show up more vividly in vibration cut sections than in frozen cut sections, and with less background. This advantage is especially noticeable with HRP reactions or immunochemistry stains, depending upon whether the antigen is in cytosol or bound to a membrane.

Vibrating microtomes are also the gentlest, most effective way to cut brain slices for electrophysiology in a tissue chamber, in part, because the tissue can be cut

under an oxygenated media. For this application, a disadvantage of vibrating microtomes is that the force applied to move the blade back and forth inevitably creates a small amount of vertical vibration of the blade (Geiger et al. 2002). This creates a layer of dead and crushed cells on the slice being cut. Various strategies for minimizing this effect are used by different manufactures. Electromagnet driven oscillation at line frequency (60 Hz) of a mass exactly correct for natural frequency of oscillation at 60 Hz has been one strategy used to minimize vertical vibration (Vibratome, Leica Biosystems, St. Louis).

The disadvantages of vibrating microtomes are that cutting is slower compared to frozen section methods, and there is a limit on how thin the sections may be cut. Fresh unfixed brain tissue can be sectioned no thinner than about 40 microns. Fixed brain can be sectioned to about 20–30 micron sections, depending on the fixation quality and hardness.

Vibrating microtomes allow adjustment of either frequency or amplitude of oscillation, forward speed of the blade, section thickness, and blade angle. Softer tissue requires slower forward speed and more oscillation (frequency or amplitude).

9.9 Freezing of Biological Tissue

In the research setting, optimal preparation and preservation is demanded to preserve morphology, genetic material and antigenicity. The method in which tissue is frozen for cryotomy can have significant impact on the results. An understanding of the physical properties of freezing tissue will help to choose the best means to freeze samples for a specific application.

When liquid water freezes to a solid, it may form either as hexagonal or cubic crystals, or as vitreous (amorphous) ice. This will depend on the rate, and cold level of freezing (Jongebloed et al. 1999). If the rate of freezing is very fast, the water will solidify without crystal formation, as vitreous ice. Freezing in crystal form expands the volume of liquid water. Water is the only substance known to have this property of expansion when freezing. Crystal formation is encouraged and larger crystals are formed by a slow rate of freezing transition from water to ice. Vitreous ice, without crystals, does not expand upon freezing. This is the basis of the often stated need to freeze biological tissue very fast, to achieve the solid state as vitreous ice. If crystals form and expand, they stretch or puncture cell membranes. Later, after sectioning and thawing the tissue, cell contents leak out into space around the cells or over the section surface. If staining for something in the cytosol such as HRP or antigens, the leaked contents may cause weaker staining in the cell and stronger background staining. If large crystals form, cells are crushed and pushed aside, and the section on the slide will be seen to have multiple holes of varying size, the well known “Swiss cheese artifact.” See Figs. 7.1–7.4.

Rapidly freezing tissue is not as uncomplicated as it might sound. Placing tissue on a pedestal and putting it in a cryostat to freeze will usually result in Swiss

cheese artifact. Therefore, in many research applications, tissue is snap frozen before being placed in a cryostat or on a freezing microtome stage. There are many methods to accomplish this. The rate of freezing will depend on several factors: The area of tissue surface relative to its volume in contact with the cold source; the intensity of cold, and the size of the tissue block. Biological materials are poor thermal conductors, so larger blocks are harder to snap freeze throughout the volume. Researchers needing to freeze whole organs such as a whole brain have a considerably more difficult task to quickly complete the freezing process, than do their colleagues in clinical histology, who more typically freeze biopsy samples or small tissue fragments.

Liquid nitrogen is the coldest fluid widely commercially available (boiling point at −195.8°C). Full immersion in cold fluid creates the most surface contact, and should therefore give the fastest freezing. However, liquid nitrogen has a very low specific heat constant. The state change from liquid to gas requires far fewer calories than for most materials, such as water. Immersion of a warm tissue in liquid nitrogen, therefore, converts a comparatively large volume of the nitrogen into gas form. The gas is an excellent insulator (as is the biological tissue) and may cling to the surface of the tissue in random patterns. Small samples can be very satisfactorily frozen upon initial contact with the nitrogen, and freezing samples this way is common practice in clinical histology labs. However, whole rodent brain will snap freeze as vitreous (will not expand) around its outer surface to some depth, but the combined effect of the gas layer building up, and the thermal resistance of the tissue, will slow the rate of penetration of the cold into the interior of the piece. Inside, the tissue may freeze slower into a crystalline form, and expand. The result is the commonly seen cracking or splitting of the tissue block, as its interior expands, and its exterior does not. If the tissue block cracks, the inside was frozen crystalline, not vitreous, and cell membranes are damaged and cell contents would have leaked in the interior, but not in the shell.

Various other liquids can be used, cooling them with liquid nitrogen or by stirring in crushed dry ice. Heat from a rodent brain will not vaporize isopentane, dry ice will not solidify it, and the specific heat constant is high enough that whole rodent brain can be reliably immersed in a slurry of dry ice and isopentane, with good vitreous ice results. The isopentane will not penetrate the tissue on immersion. Ethanol would similarly work, except that it would penetrate and react with the tissue. Isopentane is volatile and flammable at room temperature, and must be handled with care. There are a few thermal transfer fluids such as silicone oil that would theoretically work with better properties, but have not been reported as being used for that purpose.

Another effective procedure is to place the tissue on a cold pedestal and immediately bury in finely powdered dry ice. This freezes almost as fast as liquid immersion, and does not crack tissue the size of a rat brain.

The Allen Reference Atlas of C57BL6J Male Mouse by Hong (2008) describes a procedure of embedding the fresh brain and placing it on an inch thick plate of aluminum, which has been resting on a mix of alcohol and dry ice chunks, long enough to stabilize its temperature. This yields artifact free tissue that stains very well for

cytosol proteins, although completing the freezing takes minutes rather than seconds. The block can be seen to freeze at the bottom, the line of frozen tissue rises slowly. It appears that tissue above the rising freeze line is above freezing temperature and so does not start to freeze until the cold line reaches it.

If slower freezing is necessary or desired, or the tissue must be stored for some time, and if the tissue is well fixed, use of cryoprotectants may help protect tissues. Saturating the tissue with cryoprotectants such as 30% sucrose, glycerin, or polyethylene glycol offers some protection from freezing artifact. Cryoprotectants can be delivered by perfusion to save days of penetration time. Unfixed tissue is not amenable to cryoprotection because it would shrivel due to the high tonicity of these compounds. Cryoprotectants disrupt ice crystal formation, and reduce the likelihood of artifacts from ice crystals. A colder sectioning temperature is required if cryoprotectants are used. Cryoprotectants are not necessary or helpful if the tissue can be snap frozen successfully, and will be sectioned soon after freezing.

Thus, while a clinical histologist can commonly use liquid nitrogen to achieve excellent frozen tissue samples, the researcher will probably need another solution, due to the size of the tissue block to be frozen, and the specific heat constant of liquid nitrogen.

Regrettably, vitreous ice is not a stable state in nature. At any temperature, it will very slowly reform to crystalline ice (it may be years at very cold temperature), the warmer it is, the faster it crystallizes. Even vitreous frozen tissue will not last forever in the freezer, without developing artifact. Colder is better. However, tissue must be warmed in order to section, to about (−9° to −19°C). Colder cutting will leave the tissue too hard, and it will shatter when the blade bevel forces the tissue section to bend. See shattering Chap. 5. To avoid crystallization, cutting should begin as soon as the tissue reaches an acceptable temperature, not left in the cryostat overnight to stabilize. Once in the cryostat or on the microtome freezing stage, animal tissue can be sectioned in the same manner as clinical tissue, as described in other chapters.

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