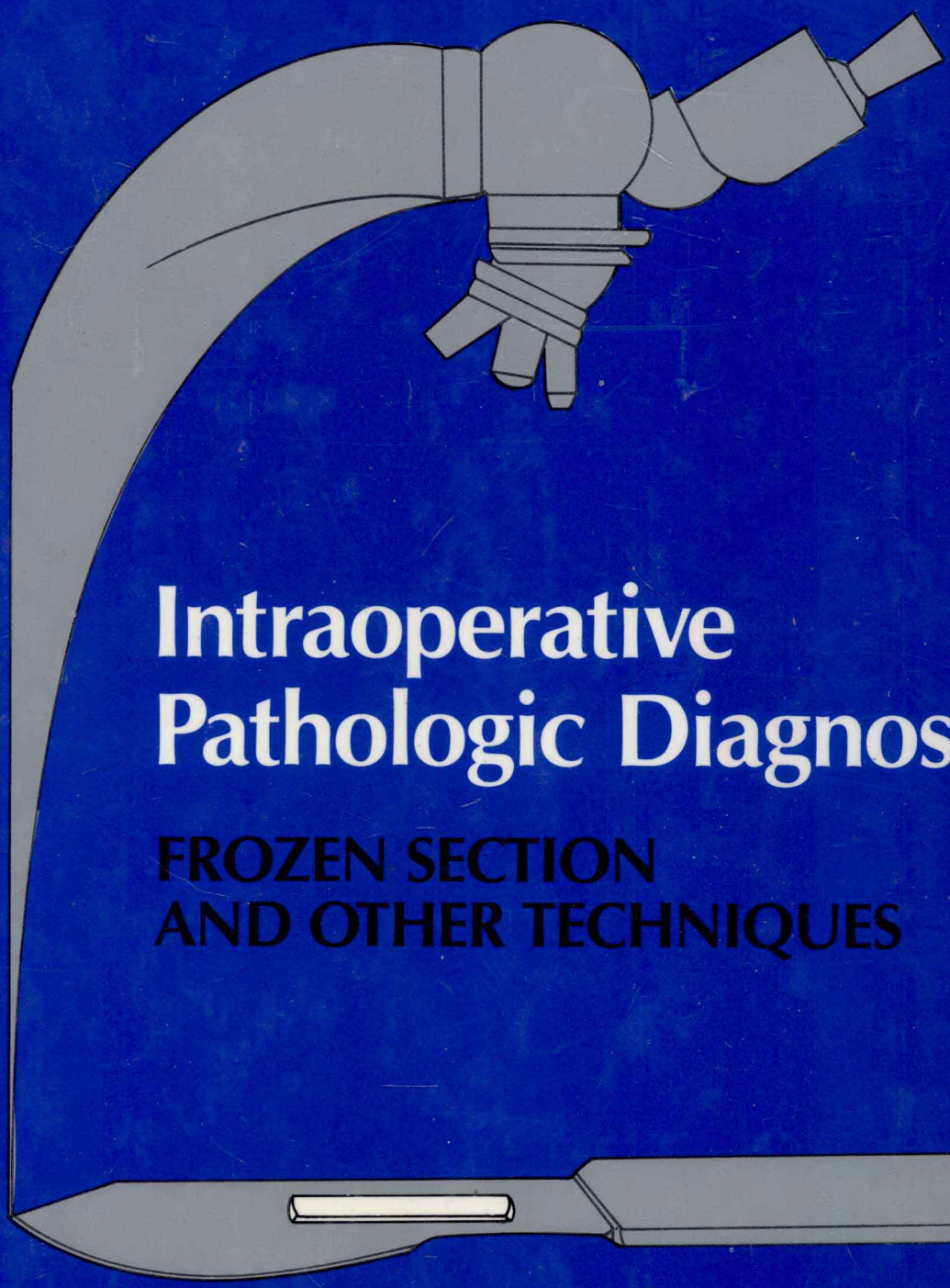

Intraoperative Pathologic Diagnosis

FROZEN SECTION AND OTHER TECHNIQUES

Elvio G. Silva
B. Balfour Kraemer

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Accurate indications, adverse reactions, and dosage schedules for drugs are provided in this book, but it is possible that they may change. The reader is urged to review the package information data of the manufacturers of the medications mentioned.

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Preface

Over the years, the traditional practice of surgical pathology has been expanded by the application of electron microscopy, immunocytochemistry, and flow cytometry. New techniques, such as DNA hybridization, and an increasing reliance upon computers will undoubtedly further expand our diagnostic abilities in the future. Amidst such technologic advances, the frozen section has remained a valid and indispensable tool by which important therapeutic decisions are made. Since the impact of a given frozen section diagnosis is often great, the diagnostic process can create stressful moments in the life of the pathologist. Surprisingly, no source of information has heretofore been compiled to assist the pathologist with intraoperative diagnostic difficulties, or to help delineate the implications of a particular frozen section interpretation.

This text is designed for use at the time of frozen section. Both pathologists and surgeons should find it a helpful reference. The fact that it is arranged according to organ systems should make for convenient access to a desired topic. On occasion, the reader will find some overlap; the same topic may be discussed in two different sections. Here, two different authors have provided commentary on their respective approaches to a given problem. In addition, there is a separate chapter devoted exclusively to neuroendocrine neoplasms. The overall emphasis placed on neoplastic proliferations reflects the nature of accessions examined at a large referral center (M. D. Anderson Hospital and Tumor Institute). Experience provided by a high volume of frozen sections has allowed us to develop what we believe is a comprehensive analysis of the problems and situations most likely to be encountered at the time of frozen section.

Our book represents a unique offering. By sharing our combined experience with frozen section diagnosis, we hope to benefit those like ourselves who are fated to struggle with the frozen section and, of necessity, must not be confounded by it.

E.G.S.
B.B.K.

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Preparing and Evaluating Frozen Tissue Sections: Techniques and Cytology

Elvio G. Silva, M.D.

The methods to be used in diagnosing frozen tissue sections will vary with the number of frozen sections needed, the staff working in the frozen section laboratory, the technicians' familiarity with a given method, and the pathologists' preferences. These are probably the reasons that no method for frozen section diagnosis has been universally accepted.

The method selected for tissue freezing is usually related to the laboratory's work load. In our department, in which an average of 75 frozen sections is performed daily and there are five full time technicians at work, we prefer to use the dry-ice box technique, a method of tissue freezing that allows us to freeze 40 blocks of tissue in two separate boxes at any given time with a high degree of safety and efficiency. In a frozen section laboratory with an average of five frozen sections a day and fewer technical personnel, an entirely different situation is presented. Under these conditions, a device that uses Freon, isopentane, or liquid nitrogen may be more efficient.

Methods of fixation, dehydration, staining, and hydration of the frozen tissue sections also depend on the technicians' and pathologists' preferences. Some centers use cytologic preparations, which in some cases might obviate the need for frozen section.

In this chapter, different methods of preparing frozen sections are discussed, and the most common problems in frozen section technique and evaluation are outlined.

INDICATIONS FOR FROZEN SECTION

The primary reasons for preparing and evaluating a frozen tissue section are as follows: *a*) to obtain a diagnosis from which a therapeutic decision may be made, such as checking the margins of resection, determining the extent of disease and type of operation needed, and identifying the tissue in question; *b*) to ascertain the adequacy of the biopsy material; and *c*) to provide tissue for ancillary studies.

Margins of Resection

Tissue sections are not usually taken to confirm resection margins when the tumor and margin of the specimen are separated by 3 (or more) cm of uninvolved tissue. When the margins must be examined microscopically, the first step is to study a representative tumor section. This will help the pathologist to better search out areas of tumor in the resection margins if he is acquainted with the features of the primary. The second step is to section the margin and avoid contaminating it with tumor cells during the procedure. To achieve this, blades are changed after the tumor is sectioned. If tumor is intentionally sectioned in continuity with a respective margin, the section should be made such that the area containing tumor is sectioned last.

An acceptable pathology report on the margins of resection includes information on the margin itself and the distance from tumor to margin; the latter is often important in differentiating between a margin and a safe margin. In endocrine carcinoma of the skin (Merkel cell carcinoma), for example, a safe margin means that a tumor is more than 2.0 mm from the margin (1). The report of a negative margin for this tumor could be meaningless if the tumor was 0.5 mm from the margin. There is no ideal method for checking margins of resection. A section perpendicular to the margin for the purposes of determining distance from tumor to margin is often performed, but such a section will usually not sample the entire margin of resection. A tissue section parallel to the margin, on the other hand, will not be informative about the distance from tumor to margin. We believe that, in most cases, sections perpendicular to the margin and from the area closest to the tumor should be taken to verify a safe margin.

In 1974, a new technique was proposed as an "exacting method for the surgical removal of cutaneous tumors" (2). This technique, a modification of Mohs chemosurgery (3), was called microscopic-controlled excision. Using this method, the surgeon removes the tumor, examines the margins of excision, and submits for microscopic examination, by means of frozen section, tissue from the convex parts of the specimen or, with a second excision, tissue from the surgical defect (4, 5). Sections are always taken parallel to the scalp blade. This technique does not provide information about the distance from tumor to margin (see also Other Indications for Frozen Section, this chapter).

Other Indications for Frozen Section

During surgical operations, frozen section is commonly used to determine the extent of the patient's disease. The information will determine the resectability of the lesion and the extent of the operative procedure. Often this indication for frozen section overlaps with checking the margins of resection.

Probably the most common situation in which frozen section is performed for tissue identification is during exploration of the neck and anterior mediastinum to search for parathyroids. Diagnostic differentiation between parathyroid adenoma and hyperplasia is often based on identification of all the parathyroid glands to determine whether the process involves one or

more glands. Since it may be difficult to distinguish parathyroid glands from lymph node and thyroid outgrowths, frozen sections help to make this distinction.

When frozen section is used to confirm the presence of the lesion in question in the biopsy material, the procedure will avoid a delay in diagnosis and a second operation. This is so for patients who have tumors with extensive fibrosis, ulceration, and necrosis and for patients whose lesions cannot be biopsied without general anesthesia or who have lesions in difficult to visualize areas, such as the nasopharynx.

Frozen sections are used also for ancillary studies of tissues, such as tissue markers in lymphomas.

Contraindications

In our experience, the only contraindication for freezing tissue is a situation in which the sample submitted for examination is so small that it is impossible to process enough tissue for permanent section. Our arbitrary minimum is 3 mm for the tissue's largest dimension, a sample size that is difficult to bisect without creating crush artifacts. Most of the time there is not enough residual tissue to make a diagnosis on the basis of the permanent section. The situation obviously varies in each case and applies with regularity only in cases without a previous definite diagnosis.

TISSUE FREEZING

The specimen received for frozen section examination should be fresh and free of excess moisture, but not entirely dry. Wrapping the specimen in gauze with saline solution may produce severe artifacts and create holes in the tissue.

During the freezing of tissue, water molecules aggregate into ice crystals, and once the nuclei of the crystals are formed, they expand and destroy all organelles around them. When the frozen section is picked up with a warm slide, the thawing ice crystals will leave holes in the tissue. The number of crystals in tissue is inverse to the speed of tissue freezing (6, 7). Thus, slow-freezing methods should be avoided.

Use of a specimen holder (tissue chuck) is a good technique for freezing tissue because the metal has high thermal conductivity. A supporting medium is needed in this situation to make the tissue adhere to the specimen holder. A few years ago, the medium of choice was saline; but now the one in widest use is optimal cutting temperature solution (OCT). This polysaccharide, a mixture of glycols and resins, is water-soluble and will rinse away during fixation and staining. Different types of OCT solidify at different temperatures: $-10°$ to $-20°C$, $-20°$ to $-35°C$, and $-35°$ to $-50°C$. We use the middle one. OCT is applied to the corrugated surface of the specimen holder (the surface promotes OCT adhesion), then the specimen is deposited on the OCT and frozen on the holder.

We use the slide method in setting the tissue on top of the specimen holder, selecting the face from which the frozen section is cut and placing this slide downward on a glass slide. The slide is then inverted on top of

the specimen holder containing OCT, and the tissue is placed on it. We prefer slides to forceps in setting tissue on top of the holder, because with this method we can be certain that the desired surface will be cut. In addition, after the tissue is set on the OCT, exerting minor pressure with the glass slide helps to create a flat surface for cutting, which will substantially reduce the need for specimen trimming. For small specimens, a cork disc can be placed between the holder and the specimen.

Rapid freezing of the tissue depends on the size of the specimen, the temperature of the cooling agent, and the rate at which heat is removed from the specimen (the three basic characteristics of heat transfer). Specimen size is very important, because all freezing techniques dissipate the heat present on the tissue surface; thinner specimens will, of course, freeze faster. The ideal thickness is probably 1 to 2 mm; and tissue to be frozen should never be thicker than 3 mm.

Tables 1.1 and 1.2 list some coolant characteristics. The ideal coolant is liquid, with a boiling point above room temperature, a freezing point as low as possible, low viscosity near the freezing point, and high thermal conductivity (8). By these criteria, isopentane is the best coolant, followed by Freon; but many laboratory workers prefer Freon because isopentane is highly flammable (ignition temperature, 420°C). In addition, near the freezing point, Freon is less viscous than isopentane.

The thermal conductivity of nitrogen, isopentane, and Freon differs significantly. Liquid nitrogen forms a "film coat," an insulated layer of vapor, around the specimen, which interferes with heat transfer. Isopentane and Freon have high thermal conductivity, because they do not form such coats.

Among these three biochemical components, liquid nitrogen is the only one that does not require a special cooling method; as long as it is maintained in a liquid state, its temperature will be between −209° and

Table 1.1.
Most Commonly Used Coolants and Different Methods of Tissue Freezing

Coolant	Temperature Obtained, °C	Freezing Time, s	Disadvantages
Liquid nitrogen	−196	10	Produces a film coat that interferes with heat transfer Frostbite Requires constant supply In nonventilated areas, N_2 may consume O_2
Isopentane	−40 to −60	25	Explosive Requires constant supply
Freon	−40 to −60	25	Requires constant supply
Dry-ice box	−60	30	Requires constant supply
CO_2 quick-freeze	−40 to −70	15	Cumbersome Requires constant supply
Quick-freeze	−30	110	
Rapid-freeze attachment or heat extractor box	−35	90	Number of specimens that may be frozen depends on brand of cryostat

Table 1.2.
Some Characteristics of Biochemical Coolants[a]

Coolant	Freezing[b] Point, °C	Boiling[c] Point, °C
Liquid nitrogen	−209	−195
Isopentane (2-methyl butane)	−160	27.9
Freon (chlorofluorocarbon)	−160	−29

[a]Indicates temperature limit between solid and liquid phases.
[b]Below this temperature the biochemical component will become solid.
[c]Indicates temperature at which a liquid will transform into a gas.

−195°C. Isopentane and Freon are maintained at −40° and −60°C by surrounding their containment vessel with a second container filled with dry ice or liquid nitrogen or by use of an electronic device that creates a cold bath with magnetic stirring. These devices reach −60°C in 30 min.

FREEZING DEVICES

To freeze tissue in the different coolants, the tissue block is immersed in the coolant container by means of special forceps. Since this could be a problem when 10 or more specimens must be frozen at the same time, we use a dry-ice box. This is a styrofoam-insulated, stainless steel box in which are located four metal bars containing five holes each, such that 20 tissue blocks per box can be frozen simultaneously (Fig. 1.1). The holes are filled

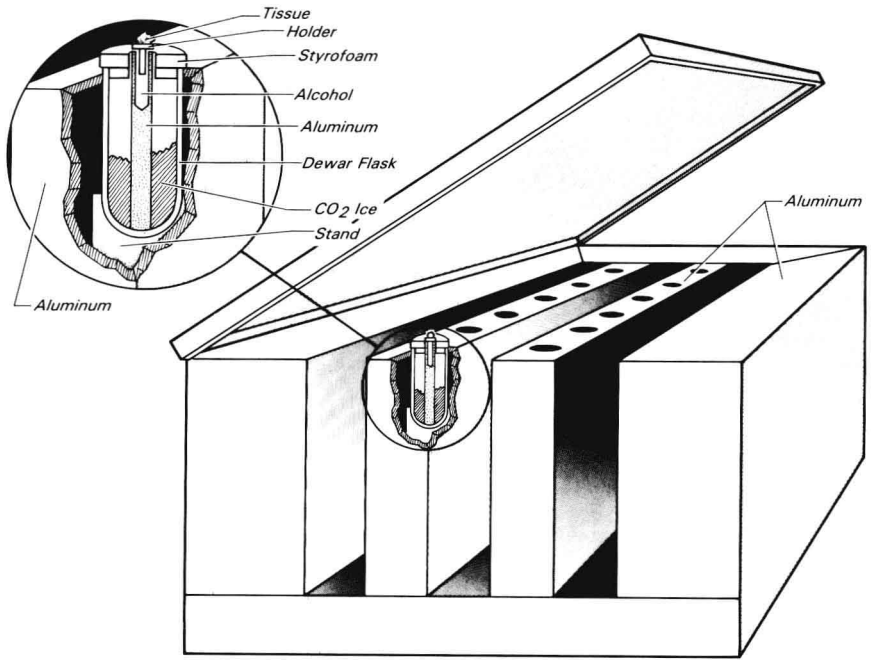

Figure 1.1. Diagram of the dry-ice box.

with 95% ethyl alcohol, which acts as a heat transfer bond (9). To freeze a tissue block, the specimen holder is set in a hole, and a small amount of OCT is laid on the holder's surface. When the OCT begins to solidify, the specimen is placed on it. A layer of OCT between the holder and the bottom part of the specimen prevents the knife from hitting the tissue holder when deeper tissue sections are needed. Placing a cork disc between holder and specimen will also eliminate this problem.

The CO_2 quick-freeze attachment is a device that connects to a cylinder of CO_2. The specimen holder is placed into the device, and the tissue is frozen immediately on contact with the CO_2. The primary difficulty with the CO_2 procedure is that the specimen must be held with forceps, because the flow of CO_2 may displace it from the holder. Some CO_2 quick-freeze attachments include a metal box designed to minimize movement. Cryostats are sometimes equipped with a quick-freeze system powered by the main processor and connected to a lateral bar inside the chamber. Freezing specimens with this system takes more than 1 min, and therefore additional artifacts will be induced.

The quick-freeze attachment, also called a heat extractor block, consists of one or two metal discs applied on top of the specimen to be frozen. The principle of high thermal conductivity is again applied in this device. Since the discs are solid metal, their temperature is probably 10° or 15°C below the temperature in the chamber; they will rapidly transmit this lower temperature to the specimen and extract the heat.

Freon aerosol spray, which is commonly used, is stored in small containers and discharged through a 5-in. flexible nozzle. Freon spray is effective when the surface to be cut in a frozen block has not hardened. In this situation the block is frozen, but, because of manipulation or friction, only a superficial part of the block is not at optimal temperature. However, freezing an entire fresh tissue block with Freon spray is difficult, expensive, and requires more than 1 min of freezing time.

Most tissues are cut well at $-10°$ to $-20°$C. Extremely soft tissue, such as brain, spleen, adrenal gland, or lymph node, is best cut at $-7°$ to $-10°$C. A very low temperature of $-20°$ to $-40°$C is needed for fat. The temperature of the frozen tissue block is usually lower than the optimal temperature at which tissue should be cut, but it will rise from the time the block is taken from the freezing device to the moment it is cut in the cryostat. When the tissue block temperature is too high, the tissue sections will become compressed, forming a mass that adheres to the knife. In this situation, the block may be refrozen by the previously used method or with Freon spray. When the tissue block temperature is too low, the tissue will fragment. In this case, the optimal temperature may be obtained by removing the block from the cryostat or applying the thumb to the top of the block.

Cryostats

Pieter de Riemer, a 19th century anatomist, was probably the first man to freeze tissue by immersing it in a freezing mixture (10). He was trying to discover a method for increasing tissue's consistency in order to obtain sections thin enough to be examined with a microscope. In 1891, Welch was one of the first pathologists to prepare and evaluate a diagnostic frozen

section (11). He used CO_2 to freeze tissue taken by Halsted (a biopsy of the breast). Wilson in 1905 and MacCarty in 1929, both working at the Mayo Clinic, gave great impetus to the CO_2 method of freezing tissue (12, 13).

Next in the development of sectioning frozen tissue was the design of a closed cabinet in which the microtome and knife could be maintained at low temperature. Linderstrom-Lang and Mogensen first attempted this in 1938 by placing dry ice inside a cabinet and using a fan to blow the cool air to the front where the microtome was located (14). When a refrigerated system was attached to the cabinet, the unit was designated a cryostat. Russel, Chang, Ibanez, and Speece at The University of Texas M. D. Anderson Hospital and Tumor Institute at Houston in 1955 were the first pathologists to use a closed-chamber cryostat in diagnostic pathology (9, 15, 16). These physicians modified the cryostat Linderstrom-Lang and Mogensen had designed for histochemical research, and they published their results in 1960 (15, 16). At the same time, Klionsky and Nunnally reported their experience with cryostats in surgical pathology (17, 18).

Two types of cryostats are used for freeze sectioning.

The Closed Unit. Tissue blocks are placed in the closed cryostat through a small door, and slides with sections are taken out, although the door remains closed most of the time. All necessary movements in the cryostat are performed with hands covered by special, insulated cryogloves, and the process is observed through at 7 × 7-in. window. The closed cryostat has some disadvantage, because the large cryogloves are clumsy, and operators need experience to operate the instrument.

The Open Top. The open-top cryostat unit has a large sliding top window through which the specimen is introduced, the microtome and knife adjusted, the section made, and the slides taken out.

A microtome that uses CO_2 is not a cryostat, because it is not a cooled chamber containing a microtome. In these CO_2 microtomes, sections tend to adhere to the knife, because it is at room temperature. The major limitations of the CO_2 microtome are the special skills necessary to obtain satisfactory sections (sections are usually thinner than 12 μm and are difficult to obtain). By use of closed or open cryostats it is not difficult to cut sections as thin as 5 μm.

The temperature at which a cryostat should be maintained depends on its use. For surgical pathology, a cryostat should be maintained at $-20°C$, because this is the best temperature for cutting tissue. Cryostats that reach $-40°$ or $-50°C$ are suitable, therefore, for diagnoses based on surgical frozen sections. If histochemical studies are to be performed on the tissue, the cryostat should be able to reach a temperature of $-80°C$. In laboratories in which only a small number of frozen sections are done, the cryostat cannot be permanently maintained at $-20°C$. In this situation, it is advisable to work with a cryostat equipped with a quick-freeze system that can drop the temperature from $-10°$ to $-30°C$ in 5 min or less.

Cryostat Maintenance

One problem with the cryostat, primarily with the open-top unit, is the difficulty of maintaining a uniform temperature throughout the chamber.

Some cryostats are equipped with fans inside the chamber to circulate the cold air, but these fans may also blow small tissue particles around. During sectioning, the knife produces a dynamic energy that is dissipated as heat, so it is important to obtain a temperature reading at the level of the blade. Some cryostats are designed to maintain a specific temperature in the specimen holder that is independent of the temperature in the chamber. When the temperature in the block rises, cryostats equipped with this system save considerable time, because it is unnecessary to cool the entire cabinet to lower the temperature in the tissue block. A main compressor cools the entire cabinet while a second compressor cools the specimen holder area. A small thermostat is located in the area of the specimen holder. Since the temperature of the knife is of paramount importance in preparing frozen sections, the knife blade should always be kept inside the cryostat.

The difference in temperature between the inside of a cryostat and the ambient temperature of the room produces a fog on the cryostat window. Windows heated by electric wires passing through them and by other means have been designed to correct this problem.

When the cold chamber of a cryostat is open to ambience it produces water molecules, which transform into ice crystals in different parts of the cabinet, primarily on the inner walls. In this situation, it is necessary to defrost the cabinet. In our laboratory, cryostats are defrosted every 3 months. Some cryostats have a defrost system connected only to areas in which ice accumulates most frequently. Since ice crystals are rarely seen on the knife, this selective defrost system protects the knife from changes in temperature. Rarely is a microtome out of operation because of the formation of ice crystals; crystals can be dissolved with drops of absolute alcohol, but the previously stiffened parts should then be dried and oiled. Proper maintenance of a microtome will avoid frosting of its parts. In our laboratory, the microtomes are completely cleaned of debris at the end of each day. They are then lubricated with a special low-temperature cryostat oil that protects moving parts and prevents frosting. Some of the adjustment wheels on the microtome are coated with nylon to protect the operator from frostbite.

Cutting Techniques

The technique of cutting tissue in a cryostat is similar to that used for permanent sectioning of tissue embedded in paraffin. The best knife angle for cutting frozen section is 30°. A vertical position of the knife should be avoided to prevent sections from falling to the bottom of the cabinet. Section thickness varies according to the type of tissue, sharpness of microtome knives, and skill of the operator. We try to obtain sections no thicker than 4 to 5 μm. In open-top cryostats, the sections tend to roll over on the surface of the knife, probably because of temperature differences in tissue, knife, and room, a problem that does not usually occur in closed cryostats. To avoid this situation, most open-top cryostats have a transparent plastic antiroll plate. This plate must be positioned immediately over the knife edge and be parallel to and approximately 1/64th of an inch from the knife facet.

The microtome knife may be cleaned with various solutions to lessen compression and eliminate static electricity. Teflon spray, antistatic spray, or solutions containing copper sulfate, stannous chloride, sodium thiosul-

fate, and sodium chromate may be used to diminish compression and friction. The most common causes of compression, however, are a dull knife, an improper knife angle, and too warm a temperature.

After the sections are cut, they may be picked up from the knife with glass slides, or transferred to a Petri dish containing water and then recovered with a glass slide. The thickness of the sections can be observed in the Petri dish. Sections that float rigidly are too thick, whereas those that fold and unfold freely are of the proper thickness. Tissue sections tend to fold over and often appear wrinkled on the glass slide. A camel hair brush or one of softer, more expensive, sable hair (our preference) is used to unfold and correct the position of the tissue sections.

Slides for use in picking up sections may be outside the cryostat at room temperature or inside the cabinet at $-15°$ to 20°C. The difference between the cold tissue section and the warmer glass slide is an important factor in the adherence of tissue to slide. We prefer to maintain glass slides inside the cryostat because sections adhere weakly to cold surfaces, allowing for easy correction of folds and wrinkles. When the tissue has flattened on the slide, it is taken out of the cryostat, and the warmer outside temperature helps the tissue adhere to the slide. This can be accelerated by holding the slide face without tissue in one's hand.

Most important in the adherence of tissue to slide is the presence of proteins in the tissue sections. Since fixation denatures proteins, frozen sections cut from fixed tissue will not adhere to slides. To correct for this, slides may be dipped in a solution of gelatin, albumin, chrome alum, and distilled water; the tissue on the slide should be air dried or placed inside a drying oven for several minutes before staining.

FIXATION AND STAINING

We stain frozen sections with hematoxylin and eosin because it preserves them indefinitely and it is a familiar technique. Our fixation and staining procedure has five basic steps (see PROCEDURES AND SOLUTIONS, this chapter; and Fig. 1.2).

1. Fixation

Time required is 30 to 50 s. We prefer to use equal parts of ether and methanol, but formaldehyde solution, 95% alcohol, or a mixture of both (one part formaldehyde at 40% plus nine parts of alcohol), or 100% acetone may also be used. The fastest fixative is methanol (20 to 30 s). For fatty tissue avoid alcoholic fixatives. Fixation will be faster if the fixative is at room temperature. To minimize artifacts, it is preferable to pick up sections with a cold glass slide and to fix the tissue in a solution containing osmium tetroxide, acetone and alcohol at a temperature below 0°C. This technique is similar to the one used for fixation of tissue by freeze substitution, in which the ice in the tissue is not melted but slowly dissolved (substituted) in a fluid solvent. The principle of this method is that ice is soluble in some solvents at temperatures far below the melting point of ice (19). This method, however, presents some problems when frozen sections are used for diagnosis. It is difficult to maintain the adhesion of a section on a cold