A Practical Guide to Frozen Section Technique

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Editor

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Preface

Frozen section technique is a valuable tool used to rapidly prepare slides from tissue for microscopic interpretation. Frozen section technique is used in a myriad of clinical and research settings. In surgical pathology, frozen sections are routinely used for rapid intra-operative diagnosis, providing guidance for our surgical colleagues. In Mohs Micrographic Surgery, the surgeon relies entirely on the frozen sections to determine the extent of the excision needed to eradicate a skin tumor. Numerous research applications rely on the frozen section technique to prepare microscopic slides utilizing a host of sophisticated morphologic, immunohistochemical and molecular methods.

Preparation of frozen section slides is a complex technical process requiring development of refined technical skills, as well as an understanding of the histology, microanatomy and pathology of the tissues being examined. Whether used for intra-operative consultation or in research, the results will hinge on our ability to achieve a high quality preparation.

The training in frozen section can vary considerably among the various subsets of practitioners. The subject is part of the curriculum in formal histology and pathologist assistant programs although much of the hands on technique is passed along at the work bench. Likewise in many pathology residency programs and research applications, training is accomplished entirely on the job sometimes with little discussion of the myriad of variables and difficulties the operator will experience along the way.

I like many pathology residents received training on the job with little more than a brief introduction to the operation of the cryostat, simple face up embedding, and to cut frozen sections using a brush. My teacher was a resident in his third year of training. From that point on it seemed that every specimen had its own set of properties. Some cut easily; some with more difficulty; some tissues would fall off the slide; and the function of our cryostat seemed to change from day to day. It also became painfully obvious that using the simple embedding methods available, I was unable to get satisfactory results in a many difficult situations. In the early days I lived in fear of exhausting precious minute samples. Over the years, through observation, experimentation and trial and error, a variety of parameters and approaches emerged which have played a significant role in my ability to prepare quality sections.

This book is intended to provide a simple yet comprehensive guide to learning frozen section technique. The authors hope to share what knowledge they have gained over years of practicing these techniques so that the newcomers will reach their goal more quickly than those of us who struggled blindly in the past.

My contributions I have written from the view point of the surgical pathologist and cover all of the steps in preparing the frozen section slide from grossing to cover slipping. The information consists of a set of methods and the details of that have proved valuable in my practice. I have tried to detail the many parameters which influence the quality of our preparations and examples of many of the aberrations that may arise.

Hoping this book will find its way into the hands of Pathology residents, I have included a discussion on interpretation of the microscopic slides in Chap. 7. The chapter discusses an approach to reading microscopic slides through careful examination, concentration and an organized plan for each specimen type. I have shared key observations about the ability to visually process information and maintaining focus and concentration. The chapter offers suggestions on dealing with difficult cases and making the most of what we have learned.

In an attempt to make this text a comprehensive guide to frozen section technique, I enlisted experts in areas outside of my experience. I am grateful for the contributions of Philip Hyam, who has spent his career in the cryostat industry for sharing his expertise and helping our readers to better understand the cryostat; Barbara Beck HT/HTL (ASCP) for sharing her expertise developed over years of practicing and teaching the histotechnology of Mohs Micrographic surgery; Charles W. Scouten, Ph.D. a noted expert in the field of neuroscience research for sharing his knowledge and expertise in frozen section technique as it applies to the animal research setting; and for the help of Catherine Susan Delia, BS.,HT. ASCP a highly experienced and knowledgeable histotechnologist for her guidance and in the preparation of our chapter on fixatives and staining.

The techniques and experiences shared in this book are those used successfully by the authors in their practice. As most of the information we have to share is derived from lifelong experience as such there are relatively few references to offer. In no way can we hope to know and cover the many different approaches used by our colleagues around the world. As so many of us have arrived at our own individual techniques and observations as a means of survival, I am certain that there are many with successful methods and ideas that differ from what we can offer. We all evolved in our own environment, taking what skills we have learned and improving on them where they were suboptimal. There may be some to take exception to what we have written. So many clever techniques are passed along at the lab bench but never find their way into our literature. I am hoping this text may encourage others to share their ideas and techniques. Together we hope to provide a body of information on frozen section technique to guide for those who find themselves immersed in this challenging field.

I would like to thank my dear friends Claudia Dorenkamp, Jan Minshew, George Kennedy and their colleagues at Leica Microsystems for their keen foresight in recognizing the valuable new technology and their help and supporting my mission

to bring these techniques to our colleagues. I am grateful to my loving wife and partner Jeannine for her unwavering support and tolerance in all of my endeavors and for rolling up her sleeves to share in the arduous task of running our little company Pathology Innovations, LLC; whose sole mission is to share better ways to help our colleagues and their patients. I would also like to thank the numerous bright young residents of the University of Medicine and Dentistry of New Jersey that I have had the privilege to help train. I could not have understood the process of learning without observing each of their unique examples. Their love and support, is more valuable to me than any reward I have known in my career. I would also like to acknowledge the dedicated and hard working histotechnologists and pathologist's assistants around the world. My pathologist colleagues and I could not begin to practice our profession without them. They are both scientists and artisans and are all too often under rewarded for the important job they perform and stresses we put them through.

I would like to dedicate this book to my late father George J. Peters. He was a man of limited education but of unlimited ingenuity. He taught me how to use tools; to make this out of that; and to *live* outside the box. Without his example, I doubt I could have gathered the information offered in this book.

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Chapter 1
Understanding and Maintaining the Cryostat

Philip Hyam

Abstract The chapter presents a user-friendly review of the main components of a routine cryostat and their function in the preparation of frozen sections of mammalian tissue. Topics covered include sectioning hints and tips and proper methods for disinfection and cryostat maintenance.

Keywords Cryostat • Microtome • Knife holder • Chamber temperature • Object temperature • Freezing shelf • Peltier element • Disinfection • Routine maintenance

Frozen sections, quick sections, in clinical terminology, intraoperative consultations, are prepared using a cryostat.

A cryostat is a cooled chamber, or cabinet that houses an instrument to section frozen samples; a rotary microtome and knife (or blade) holder, and a means to freeze samples.

Several types of cryostats are commercially available and can be categorized as follows:

- Single compressor (chamber cooling only)
- Double compressor (chamber and object cooling)
- Manual sectioning
- Motorized sectioning

These are free-standing instruments that are insulated to very high standards to ensure that selected temperatures are easily maintained. Access to the chamber is via a heated sliding window. The normal working chamber temperature is from 0°C to −35°C, the limiting factor being the type of compressor and refrigerant used. Cryosectioning at temperatures lower than −35°C requires the use of a cryogen such as liquid nitrogen.

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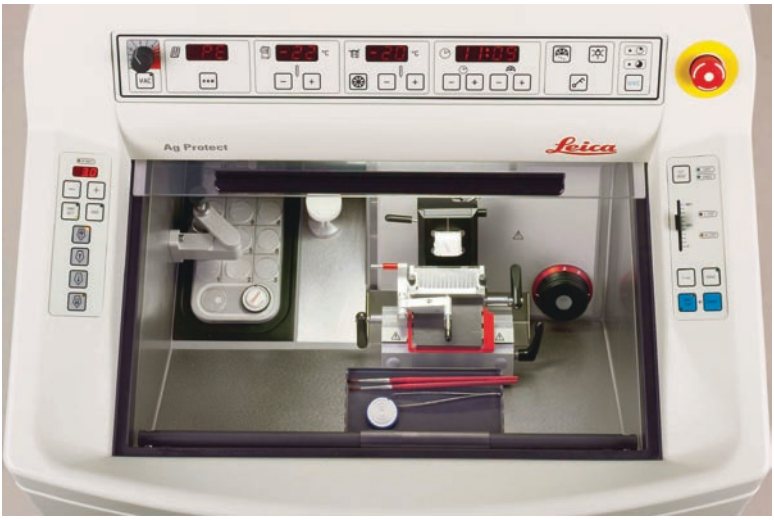

Fig. 1.1 This shows the external controls and chamber of the Leica CM1950

The rotary microtome, (Fig. 1.1) controlled by an external handwheel that is mounted inside the cryostat cabinet, has been specially manufactured and lubricated to work at low temperatures and to provide a mechanism for advancing a specimen toward a fixed knife (or blade) in precise reproducible increments with a section thickness range from 1–100 μm or higher.

To the side of the microtome is an area known as the freezing shelf that as the name suggests is the area where samples are frozen or frozen samples are stored prior to sectioning. The working temperature of the shelf averages −10°C lower than the set chamber temperature due to its location close to the compressor system (Fig. 1.2).

Newer instruments also incorporate a Peltier freezing stage with the freezing shelf (in the above illustration the Peltier stage is outlined in white). A Peltier stage is a thermoelectric device that when activated increases the diffusion of heat away from the sample to the cold stage, resulting in a higher cooling rate and thus faster freezing of the sample.

Located directly in front of the microtome and fixed either to the microtome base or the cabinet is the knife (or blade) holder. There are two types of knife holder that provide a means of clamping either a disposable blade or a reusable steel knife. Figure 1.3 shows the cryostat stage with the antiroll mechanism in place in front of the blade holder. The blade holder contains a blade clamped in position in Fig. 1.8.

Disposable blades, commercially available in either high or low profile, are supported on a ridge on the rear pressure plate of the knife holder and clamped firmly into place by a spring lever-operated front pressure plate. It is important that the

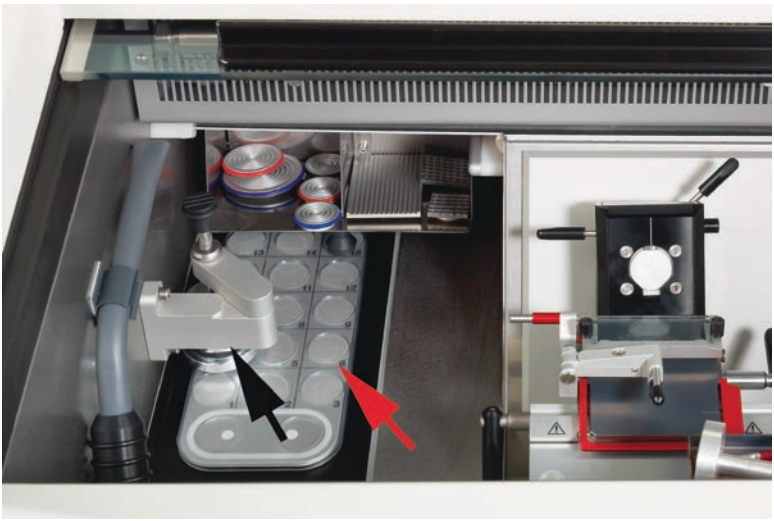

Fig. 1.2 This shows the freezing shelf on the left side of the cryostat (*red arrow*). The heat extractor is attached to the hinged arm attached to the left wall

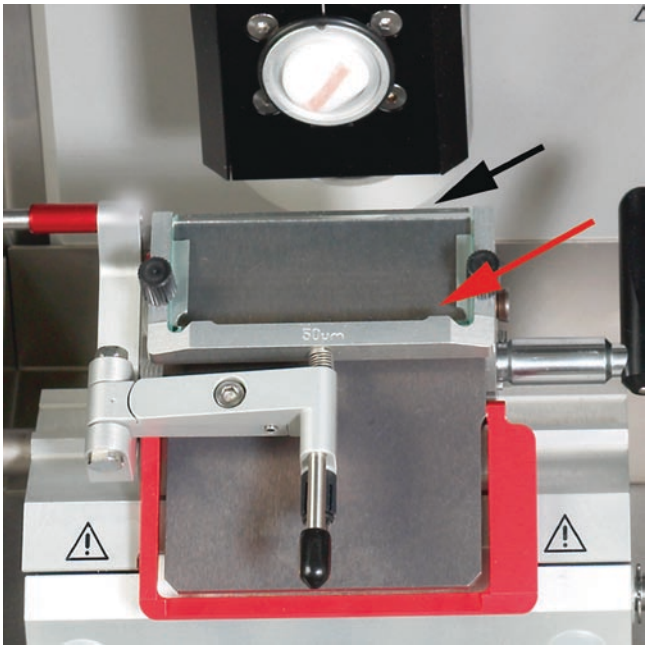

Fig. 1.3 Cryostat stage of the Leica CM1950. The knife holder (*black arrow*) is directly in front of the antiroll device (*red arrow*)

clamping pressure be maintained evenly across the entire length of the disposable blade. Damage to either the front or rear pressure plate, or section debris trapped between the pressure plates, will significantly affect the clamping pressure and thereby sectioning efficiency and quality.

An alternative to disposable blades is a steel reusable blade. Steel blades available in either C profile (steel) or D profile (tungsten carbide) rest on a support bar between two pillars of the standard knife holder. At the top of each pillar is a securing screw to ensure that the blade is clamped firmly.

Both the disposable and standard knife holders permit adjustment of the clearance angle for the blade being used. In general, for low-profile disposable blades, the blade angle is between 3° and 5°: for high-profile blades it is between 5° and 7°, and for reusable blades it is between 5° and 7°.

Both types of knife holder are fitted with antiroll guides, which as the name suggests help to prevent rolling or curling of sections as they are being prepared. The antiroll guide consists of a glass plate supported in a metal (aluminum) frame. The frame provides a gap between the underside of the glass and the upper surface of the front pressure plate or knife surface allowing the section to slide under. Gap sizes of 50, 100, and 150 μm are available depending on the section thickness required. For example, if sections of <5.0 μm were being collected then a gap size of 50 μm would be suitable.

An alternative to the antiroll guide, is the cooled brush (a fine artist's brush) technique to collect and gather sections. With this technique; as the leading edge of the section starts to come over the blade or knife edge the brush is used to pull/guide the section onto the pressure plate or down onto the front surface of the blade. The brush technique is discussed in detail in Chap. 4.

Specimen holders or *chucks* for cryostats are available in a variety of shapes and sizes.

The presence of a stem or other specially designed projection on the bottom or side of the chuck provides a foothold to clamp the chuck and is often specific to the maker of the cryostat (Fig. 1.4).

Before beginning sectioning it is important to ensure that all operating parameters have been set properly. A checklist is included at the end of this chapter.

Fig. 1.4 Specimen holders or chucks. Specimen holder of different sizes and shapes are made to accommodate various embedding tasks

1.1 Operation of the Cryostat

Cryostats must be located in a draught-free, humidity-controlled area, with clearance of 30 cm, on all sides to ensure that there is unrestricted air movement around the instrument. Failure to provide ample clearance can result in poor cooling performance, as airflow to the compressor(s) is restricted.

All accessories; knife holder, antiroll guide, brush trays, etc. should be placed into the chamber prior to the instrument being turned on. At start-up the chamber (and object temperature if applicable) temperature should be set a recommended setting is −20°C. Allow at least 24 h for a cryostat to come down to selected operating temperature.

Other settings at start-up are; time of day and defrost time. All commercial cryostats use a regular, once every 24 h, automatic defrost cycle to ensure that the chamber, microtome, and accessories are kept free of frost. During the defrost cycle the compressor(s) goes into a "hot" gas phase and for a preset period of time circulates warm air into the chamber. Any frost or ice present on the chamber surfaces is melted and the fluid is drained into a sealed container. For the majority of cryostats the defrost time is usually set to midnight; this ensures that the defrost procedure is completed and the instrument is cooled down to the set operating temperature outside of normal working hours.

In a busy hospital where frozen sections are commonly performed at all hours, it is best to have several cryostats to stagger defrost cycles to assure that one cryostat will be functional at all times.

1.2 Specimen Preparation

This section will include a general discussion on use of the cryostat. See Chaps. 4 and 5 for detailed discussions of embedding trimming and cutting. For clinical users it is assumed that institutional policies such as Body Substance Precautions (BSP) are in effect and that all operators conform to this method of practice. Protecting the operator is the prime goal of BSP, and the risk of potential infection is extremely high when working with unfixed pathological tissue.

Specimens for frozen sectioning can be prepared in a variety of ways. They can be frozen in a cryogen mixture such as isopentane cooled by dry ice, or placed onto a thin layer of cryocompound on a specimen holder that is placed on the freezing shelf, Peltier stage, or a large cooled metal block placed inside the cryochamber.

The aim is to freeze the specimen as fast as possible to eliminate ice crystal formation (freeze artifact). To achieve fast freezing the size of the specimen must be carefully controlled, as a guide a suitable specimen would be 2 cm ×2cm×2mm.

Plunge-freezing into cooled isopentane is a fast, reliable and reproducible method of freezing tissue. A small beaker (100 ml) of isopentane is placed into a

Fig. 1.5 Heat extractor mechanism

container of dry ice (solid $C0_2$), to give a working temperature of ~−70°C. Specimens frozen in this manner can then be sectioned immediately or stored at −80°C.

Frozen specimens are mounted onto a specimen holder with cryocompound and placed on the freezing shelf and allowed to stabilize at chamber temperature.

Fresh specimens can be frozen directly onto a specimen holder that is placed onto the freezing shelf. A heat extractor can be used to aid freezing and ensure good adherence and flat front block surface. A heat extractor is a large piece of stainless steel that is supported on a hinged arm that is usually mounted above the freezing stage (Fig. 1.5).

As the surface of the specimen starts to freeze the heat extractor is gently lowered onto the top of the specimen and allowed to remain there for a few minutes.

Before mounting the specimen onto the microtome, ensure that the handwheel is in the locked position. *Never* attempt to place or remove a specimen from the microtome without ensuring that the handwheel is locked. In the unlocked position the object head can move when inserting the chuck, placing the operator at the risk (Fig. 1.6).

The specimen holder is clamped firmly onto the object head mounted on the spindle of the microtome (Fig. 1.7).

The handwheel is unlocked and the specimen is lowered until it is at the same height as the knife holder. The knife holder can then be unlocked and moved manually toward the specimen until it is close to the specimen surface. In some instruments a motorized coarse advance allows the spindle/object head to be moved close to the knife.

Fig. 1.6 Cryostat handwheel. Each turn of the wheel advances the tissue block the precise increment of section thickness setting. The *arrow* indicates the locking mechanism

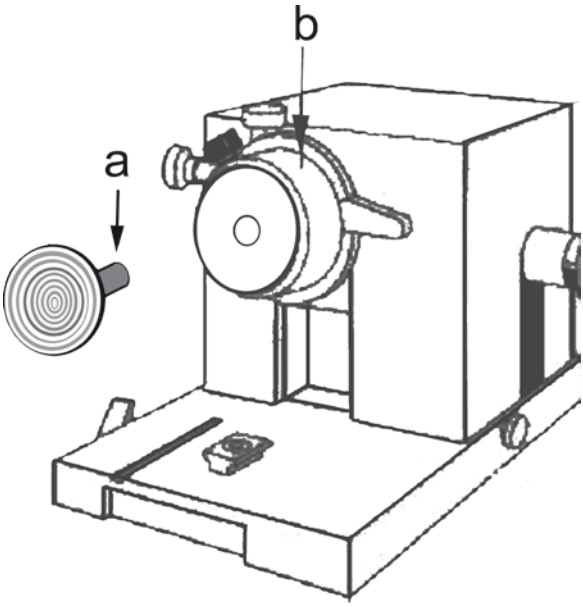

Fig. 1.7 Microtome portion of the cryostat. Letter (A) points to the specimen holder or chuck that is inserted (*arrow*) into the object head (B) mounted on the spindle of the microtome

Adjust the micrometer setting of the microtome to "trimming" thickness of 15 μm and begin to turn the microtome handwheel; the specimen will advance by this set value and will make contact with the knife and the surface of the block will be sectioned. This process is termed "trimming" or "facing" the block, and the purpose is to achieve a full face section of the specimen. As soon as this is achieved stop sectioning and adjust the micrometer setting to the desired section value, e.g., 5 μm. Brush all trimmings from the blade, or knife edge, carefully lower the antiroll plate into place and continue sectioning.

Carefully lift the antiroll guide and with a cold brush arrange or move the sections on the pressure plate or knife surface. Take a glass slide and holding it at an angle gently lower it onto the section as shown in Fig. 1.8. Retrieving tissue from the stage is discussed in greater detail in Chap. 4 (p 17).

Sections of fresh frozen tissue will adhere to plain glass slides due to the presence of free protein and lipid. Sections of fixed frozen tissue will need to be mounted on coated slides, e.g., poly-L-lysine.

Fig. 1.8 The figure shows a tissue section being retrieved from the cryostat stage. The antiroll mechanism has been reflected to the left. The *red arrow* points to the blade clamped in the blade holder. The operator must be aware of the proximity of their hand to the blade at all times to avoid injury when retrieving sections

Sections of fresh frozen tissue should be fixed immediately unless they are going to be stored for future study. A standard histology fixative such as 4% neutral buffered formalin is the most suitable fixative for frozen sections.

Sections of fresh frozen tissue will rapidly dry if exposed to warm air, and this will result in cellular artifact.

If sections, mounted on glass slides, are going to be stored for future study they should be placed immediately into a commercial (plastic) slide box that has been cooled and kept in the chamber. At the conclusion of section collection the slide box should be closed and the entire box wrapped in a double layer of aluminum foil, labeled, and moved immediately into a −80°C freezer. Tissue sections will not deteriorate as long as they are kept at a temperature of −80°C or lower.

Any remaining specimen can be stored for future use. The specimen is carefully removed from the specimen holder either by using a spatula, or by gently warming the holder so that the still frozen block can be removed. Remove as much cryocompound as possible from around the specimen; wrap it in a double layer of aluminum foil; label and store at −80°C.

1.3 Specimen Orientation

Many specimens need to be precisely oriented for adequate examination. Chapter 3 offers a detailed discussion of embedding and orientation of tissues using freezing temperature steel well bars (see p. 9).

1.4 Cryostat Disinfection

For clinical users good hygiene of cryostats is a must. Specimen shavings should be removed after each use. The waste tray that is underneath the microtome spindle should be carefully lifted out and the shavings/debris placed into a biohazard bag. Other shavings and specimen debris can be wiped up using either paper towel or a gauze swab soaked in 70% alcohol. During this procedure it is important to minimize aerosols and to prevent sectioning debris from spilleing onto open surfaces.

Disinfection systems are now offered by several cryostat manufacturers: A disinfectant spray system (MICROM), formaldehyde vapor (Thermo Fisher), a combination of disinfectant spray and UV light (Leica), and ozone (Sakura). Each of these systems uses a different method to achieve a safe working environment; users are encouraged to thoroughly investigate the efficacy of each system prior to purchase.

All users should be responsible to ensure that the cryostat is kept clean, to minimize the risk of infection by inhalation and to reduce the risk of cross-contamination of a section or tissue sample.

1.5 Cryostat Maintenance

Like all well-used instruments the cryostat requires regular maintenance to ensure optimum performance, and the tasks can be divided into daily, weekly, monthly, and annual tasks.

1. Daily:
    - Lock the microtome handwheel.
    - Remove all section debris, using either alcohol-soaked paper towel or a suitable vacuum system.
    - Carefully remove all specimen trimmings.
    - Remove all specimens.
    - Remove all blades or knives.
    - Remove all used specimen holders and soak in warm soapy water to ensure that all cryocompound is removed; air or oven dry.
2. Weekly:
    - Empty fluid container (this fluid is collected during the daily defrost cycles). For clinical pathology laboratories this fluid *must* be regarded as potentially biohazardous and disposed according to institutional policies.
    - Thoroughly clean and lubricate all contact surfaces of the knife holders.
3. Monthly:
    - Spindle lubrication: ensure that the handwheel of the microtome is locked, using the coarse advance bring the microtome spindle (object head) forward.
    - Using a disposable pipette place a few drops of low-temperature oil (supplied with the cryostat) onto the rear (close to the microtome housing) upper surface of the spindle
    - Retract the spindle to its home position. This will ensure that the spindle is kept well lubricated and free of dust debris. Spindle issues can result in poor sectioning and thick and thin sections.
    - Check the side compressor vents to remove all dust etc.
4. Annual:
    - Have the instrument serviced by manufacturer trained and approved service engineers

1.5.1 Checklist

- Prior to starting sectioning check and confirm the following
- Confirm that chamber temperature (and object temperature if applicable) is set; a recommended starting temperature is −20°C.

- Confirm that the hand wheel is LOCKED.
- Check that the knife holder clearance angle has been set correctly.
- Insert fresh blade and check that the blade is clamped firmly.
- Insert the specimen and ensure that it is clamped firmly into the object holder.
- Confirm that the desired section thickness has been set.
- Unlock the handwheel and start sectioning.
- When sectioning is completedLOCK the handwheel.
- Remove the specimen from the object holder process for either low-temperature storage or fixation for subsequent paraffin embedding.
- Remove the blade.
- Remove ALL trimmings and specimen waste.
- Ensure that the cryostat is left in a clean and safe condition.

Chapter 2
Gross Examination of Tissues in the Frozen Section Room

Stephen R. Peters

Abstract The chapter discusses gross examination of tissues in the setting of surgical pathology intraoperative consultation; *the frozen section room*. The process of gross examination is discussed in nine important steps from verifying of labeling and review of clinical information through gross examination, inking, sectioning, and cytologic preparations. These steps provide a checklist of considerations, as we perform this detailed and important part of our frozen section room task under the constraints of time. Emphasis is placed on the importance of understanding and correctly inking resection margins. The chapter offers a number of technical approaches to inking, and sectioning, as well as pointing out potential sources of error. The importance of cytology specimens and techniques for preparations are discussed.

Keywords Cytology preparations • Crush preparation • Scrape preparation • Touch preparation • Dissecting • Lymph node adipose tissue • Gross examination • Gross sectioning of tissue • Inking resection margins • Application of ink • Multiple sections • Perpendicular margin sections • Resection margin false resection margin • True resection margin • Inking resection margins • Sampling errors • Shave margin section • The sausage trick

When initiating a new resident to frozen section technique I like to start by asking the question "*What is the most important thing we do in the frozen section room*?" The answer is "*The gross*." Possibly a slight over statement, it serves to emphasize the importance of the gross examination. Our gross diagnostic acumen will never serve us better than in the frozen section room. A thorough gross examination and sampling of a wide range of tissues is our best defense against sampling errors. Recognizing important gross features of a pathologic process can strongly aid in the differential diagnosis we are considering microscopically.

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