Chapter 6
Fixation, Staining and Coverslipping of Frozen Section Slides

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Abstract This chapter covers fixation, staining and coverslipping of frozen section slides. A number of fixatives and fixative cocktails in use are described and illustrated as well as artifacts resulting from prolonged drying. Several commonly used staining procedures are described with suggestions on maximizing the quality and information gathered for interpretation. Adhesion of tissue to glass slides and causes of poor adhesion is discussed. Problems arising in the coverslipping process are discussed and method for coverslipping finished slides is offered.

Keywords Acetone • Adhesion • Air bubbles • Air dried • Coverslipping • Ethanol • Fixation • Formalin • H&E stain • Methanol • Oil red O stain • Staining • Toluidine blue stain • Wiping slides

6.1 Fixation of Frozen Section Slides

For intraoperative consultation or rapid diagnosis of a pathologic specimen, most pathologists will want their frozen section slides to resemble the preparations they are accustomed to reading on their routine paraffin embedded sections. This will require immediate fixation. There are a number of fixatives in use which include 95% ethanol, methanol, formalin, acetone and various cocktails of ethanol or methanol with formalin and acetic acid. I have experimented with a number of these. The various formulations used were obtained from polling the highly experienced technologists on the Histonet list server, an extremely valuable message board for the Histology profession that is managed as a service to the field of Histology by Dr. Linda Margraf, Dr. Herb Hagler and the University Of Texas

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S.R. Peters (ed.), *A Practical Guide to Frozen Section Technique*,
DOI 10.1007/978-1-4419-1234-3_6,

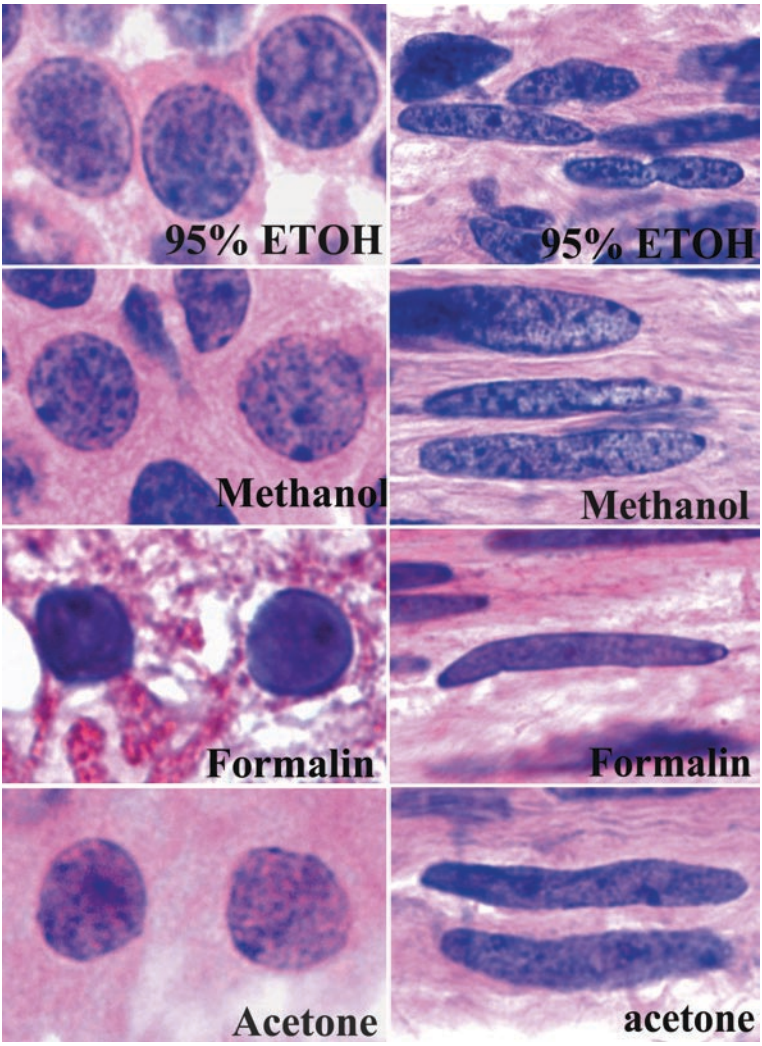

Fig. 6.1 Frozen section fixatives, single agent. The left column shows photomicrographs of renal tubular cells at 1,000× magnification. The right column shows photomicrographs of a low grade leiomyosarcoma at 1,000× magnification. The first through forth row tissues were fixed in 95% ethanol, methanol formalin and acetone respectively and are labeled on individual pictures. Both the ethanol and methanol show sharp well defined chromatin and nuclear membrane features. The formalin fixed tissues show a more smudged, less defined chromatin. Acetone showed better definition than the formalin but les definition than the two alcohols

Southwestern Medical Center Department Of Pathology. The resulting slides were all quite satisfactory for interpretation. In my experience 95% ethanol gives excellent results after only a few seconds of immersion. The most critical point is to fix the slides as rapidly as possible once the tissue is placed on the slide. The composite Figs. 6.1 and 6.2 show examples of renal tubular cells and cells of a low grade

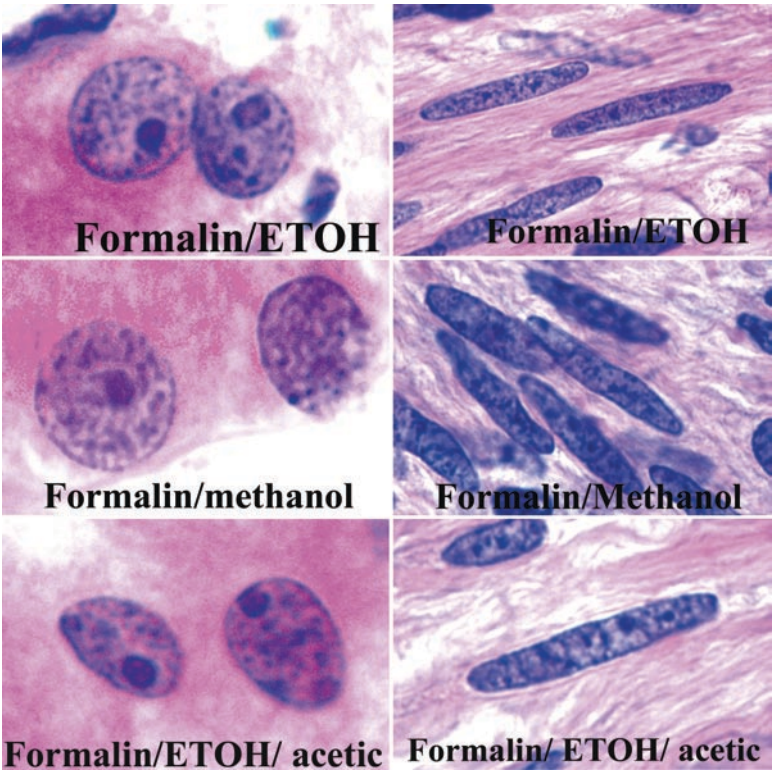

Fig. 6.2 Frozen section fixative cocktails. The left column shows photomicrographs of renal tubular cells at 1,000× magnification. The right column shows photomicrographs of a low grade leiomyosarcoma at 1,000× magnification. The first through third row show samples fixed in cocktails of formalin and ethanol, formalin and methanol, and formalin, ethanol, and acetic acid. All of these cocktails offer excellent cytologic detail

leiomyosarcoma stained with a variety of fixatives solutions. The examples showing ethanol and methanol alone and in combination with formalin and acetic acid all show sharp nuclear detail. The formalin and acetone alone yielded a slightly more smudged and less defined chromatin appearance.

The examples above represent fixative cocktails. Formalin ethanol and methanol combinations used contained 30% neutral buffered formalin and 70% ethanol or methanol solution. Formalin ethanol acetic combination contained a solution of 70% ethanol with 5% acetic acid and 4% neutral buffered formalin. If one compares these results to the examples showing air drying in Fig. 6.3, it is clear that the differences in the various fixatives pale in comparison to the artifactual changes of air drying.

From the time the tissue section or cytologic specimen touches a warm slide, it starts to develop a *drying* artifact. I consider the histological features of fixed preparations and air dried preparations analogous to an egg in its shell and in the frying

pan. Drying causes enlargement of cells and nuclei, smudging of nuclear detail with loss of contrast, leakage of fluids from the cytoplasm and blurring of cytoplasmic borders. It is as if the cells were melting and spreading on the slide. To achieve the best cytological detail, fixation should be carried out immediately. If one delays fixation for more than a few seconds there will be noticeable drying artifact. Figure 6.3 compares tissues fixed immediately with the same tissue fixed after 15 s sitting on a warm slide. The differences are dramatic. As soon as the section is complete, immediately pick up the tissue on the slide and as quickly as possible place it into fixative. Have your fixative jar opened and immediately accessible.

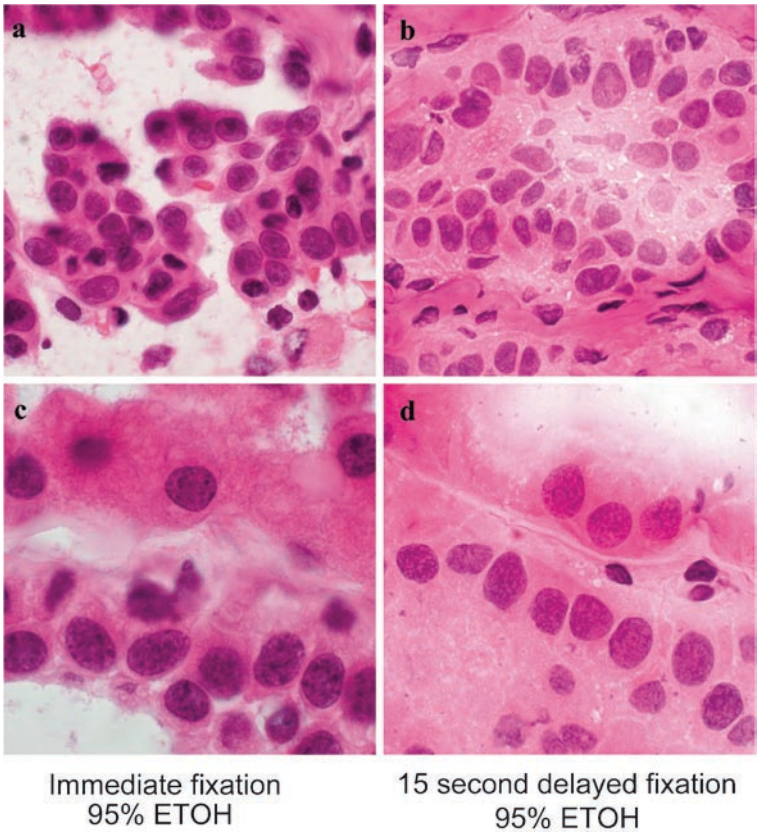

Fig. 6.3 Drying artifact. Figures (**a**) and (**b**) are micrographs of pulmonary bronchiolo-alveolar adenocarcinoma at 200× magnification. Figure (**c**) and (**d**) are micrographs of real tubular tissue at 400× magnification. The sections on the left were immediately fixed in 95% ethanol. The sections on the right were fixed after a delay of 15 s after retrieving the tissue onto a room temperature slide. Examples of the immediately fixed tissue (**a**) and (**c**) show sharp cytologic details including distinct nuclear chromatin and nuclear membrane, and well defined cytoplasmic borders. In contrast the sections with delayed fixation show smudged chromatin and poorly defined nuclear membrane. Cytoplasmic borders are blurred and give the impression that fluids are leaking from the cells as the tissue appears to melting on the slide

When using a staining rack, I suggest keeping it outside the fixative jar so it does not impede your swift motion and risk breaking the slide. First fix the slide for a few seconds in 95% ETOH then put it in the rack.

6.2 Air Dried Preparations

There are many setting where we will want to take advantage of the effects of air drying slides before fixation. Air drying frozen section slides will allow the sections to better adhere to the slide and is a requirement for many research applications where more harsh and complex staining procedures will cause greater tendency for the tissue to come off the slide during staining.

As discussed in Chap. 2, air dried cytology preparations stained with the diff quick stain will provide a new set of morphologic features to augment our fixed H&E stained sections. The expanding *fried egg* phenomenon offers a level of magnification allowing cells to be visualized at lower magnification. Cytoplasmic features are magnified. The diff quick stain offers specific staining of mucosubstances and other elements which will be more subtle or not visible on H&E stain.

6.3 Staining of Frozen Section Slides

Slides prepared by frozen section technique can be successfully stained by many of the staining procedures used for routine paraffin embedded tissues. In research applications there are a number of settings where frozen section slides are preferred due to better preservation of cellular elements. For intraoperative consultation we are limited to rapid staining procedures due to the limitation of time. The stains most commonly for intraoperative consultation are a rapid H&E (hematoxalin and eosin) stain and toluidine blue stain.

Many pathologists are most comfortable using an H&E stain for frozen section slides because it most closely resembles the preparations seen in our routine sections. Hematoxylin stains are either "regressive" or "progressive." The slides in a regressive hematoxylin stain are stained in the hematoxylin solution for a set period of time and then taken back through a solution such as acid–alcohol that removes part of the stain. This staining method works best for large batches of slides to be stained. The slides in a progressive stain are dipped in the hematoxylin until the desired intensity of staining is achieved, such as with the frozen section (Klatt and Edward 2008). Compared to the toluidine blue stain which takes a few seconds to prepare a rapid H&E will take a few minutes. Like many others, I am happy to spend the extra time to have a familiar picture to evaluate during the challenges of intraoperative consultation.

When reading frozen section slides, pathologists make the large part of their observations at scanning powers i.e., 20× or 40× magnification. At these low magnifications, a poorly stained slide offers only a washed out image, opening up possibilities

for misinterpretation and overlooking minute findings. When looking at lymph nodes for metastatic disease, it is essential to have deep rich staining, especially the eosin. If the eosin stain is rich, the pale pink cytoplasm of a sinus histiocyte can be more easily distinguished from the tumor cell cytoplasm which may be more eosinophilic or clear. With a poorly stained slide, the shades of pink and blue are not as clearly differentiated and subtle differences are lost.

6.4 H&E Staining Procedure

Hematoxalin 60 s
Rinse in water
Ammonium hydroxide % 10 s
Rinse in water
Eosin 15 s
95% ETOH 10 s
100% ETOH 10 s
100% ETOH 10 s
Xylene 10 s
Xylene 10 s
Apply cover slip with mounting medium.

When motivated by our surgical colleagues, it is tempting to move quickly through the staining rack. The extra minute it takes to properly stain a slide will make the job of reading it far easier. Do not rush any step of the staining process and to keep all stains and solutions fresh and well maintained. Gentle agitation will aid the staining process by keeping the staining uniform, but the type of tissue and its adhesive nature should be considered. Do not over agitate the tissues that are loosely adherent (*see* below).

Using H&E stain, one would expect that simply setting a timer for a given time interval in hematoxalin would yield the same result every time. Unfortunately in a busy frozen section practice, solutions can become diluted and at times may not always be perfectly maintained. Get in the habit of looking at the section as it leaves the bluing agent. Observe the shade of the section and compare it with the result under the microscope. Eventually you will get a sense for the color and shade of a well stained slide versus a lightly stained slide. There is a specific navy blue tone that tells me the section is well stained. Keep in mind; both the thickness of the tissue and the density of nuclear material will make the color to the tissue appear lighter or darker. A lymph node densely packed with dark staining nuclei will appear quite dark after the bluing agent and a piece of muscle which has widely separated nuclei will appear very light. The actual tone of blue will be similar when optimally stained. By checking the color after the bluing agent one can return the slide to the hematoxalin for additional time if necessary. Make your own observations and get in the habit of grossly checking the slide before continuing on with the staining.

6.5 Toluidine Blue

Toluidine blue stain is a commonly used alternative used for staining the frozen sections. A metachromatic stain, toluidine blue delineates the architecture in varying shades and intensities of blue and purple. A significant advantage of the toluidine blue stain is that it works almost instantly. A drop of stain is applied over the section and the slide is cover slipped and ready to read. It will stain mucosubstances faint purple and notably mast cells will have purple granules. A disadvantage of toluidine blue is that there is considerably less differential staining of the tissue. It is my feeling that we should stain the slides with the stain most familiar to us. As a pathologist, I am used to recognizing microanatomy in the familiar reds, blues and purple of an H&E stained slide. Personally, I do not think it is worth using a less familiar stain with less differential staining to save 2 min of time. I am certain that many toluidine blue users will argue this point and find that their experience with toluidine blue has made them equally comfortable recognizing the morphologic findings under the microscope.

6.6 Special Stains for Intraoperative Consultation

As I mentioned above, there are many special stains which can be applied to the frozen section slides. Unfortunately due to the time constraints for intraoperative consultation, we are quite limited to stains that can be completed in a few minutes. The oil red O stain for fat can be performed quickly although its uses are limited. Oil red O stain is used effectively in some laboratories to provide information about the lipid content of parathyroid lesions as an aid to differentiate the hyperfunctioning parathyroid glands which contain minimal intracellular lipid when compared with the suppressed normal glands which have increased lipid stores. Oil red O stain can also help recognizing tumors known to have cytoplasmic lipid as in some renal tumors.

Frequently, we are asked to look for evidence of infection in frozen section slides. These are often in immunosurpressed patients who are very ill and need the fastest possible answers. If you suspect an infectious process, take a few extra frozen sections on charged slides if possible. These can be brought to the histology lab and stained for fungi and acid fast bacilli making the results available in just a few hours depending on your labs capabilities. Figure 6.4 shows an example of a frozen section of an open lung biopsy specimen removed on a bone marrow transplant patient. The H&E section showed a few subtle areas of foamy appearing exudates. An unstained frozen section was brought to the lab and placed on an automated stainer. Within 2 h, the methenamine silver stained slide demonstrated conclusively that the pneumocystis carinii organisms were present.

Protocols for immunoperoxidase staining can also be modified for frozen section slides, opening additional possibilities for identifying viral infections such

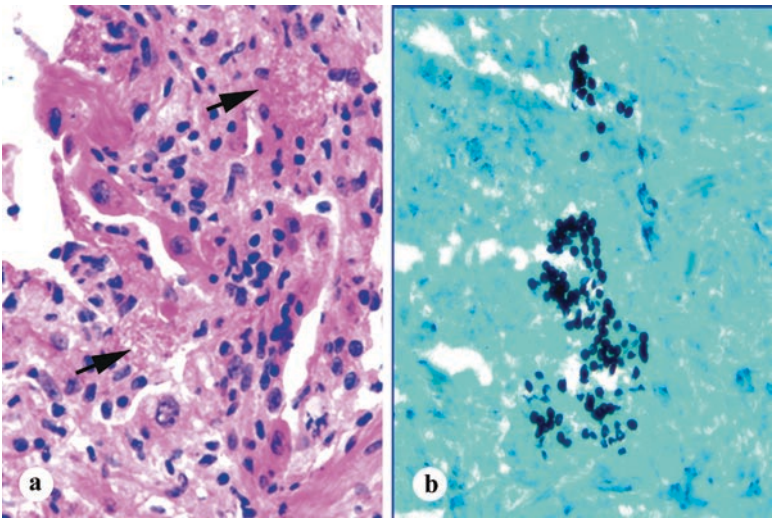

Fig. 6.4 Open lung biopsy frozen section. Picture (**a**) shows an H& E stained micrograph at 200× magnification with evidence of interstial pneumonia. The arrows point to several small intra-alveolar exudates with a *foamy* appearance. Figure (**b**) shows an additional frozen section slide taken intraoperatively and stained with methenamine silver stain on an automated stainer in the routine histology lab. The section demonstrates typical organisms of Pneumocystis carinii and was available to this critically ill patient after a staining delay of less than 2 h 200× magnifications

as cytomegalovirus, herpes virus, and adenovirus rapidly in the immunocompromised patient.

If one looks to the literature, there are reported protocols for rapid microwave staining techniques for PAS (periodic acid shiff) (1) and mucicarmine (2) staining for intra operative consultation.

6.7 What Holds Our Tissue to the Slide and Why Does It Fall Off?

Occasionally, one will go through the process staining the slides only to find the tissue has completely or partially fallen off the slide. In order to combat this problem it is worth considering the forces that hold the tissue to the slide and the forces considered that will pull the tissue from the slide.

What holds the tissue to the slide? In an excellent paper by J. A Kiernan, (Kiernan, IA 1999) it is suggested that the adhesion of tissue placed on a clean glass slide may be the result of non-ionic forces such as van der Waals forces and hydro-

gen bonds creating an attraction between the glass slide and the section. In addition, ionic attractions may also exist between the silicate of the glass (negatively charged ions, especially in an alkaline medium) and basic groups of proteins in the tissue (positive ions, especially in an acidic medium).

In my experience which consists entirely of handling fresh tissues for intraoperative consultation, I have observed that some tissues will adhere better than others. I like to think of these tissues as having more *glue.* Using positively charged slides or slides coated in polymers such as polylysine are alternatives which will offer better adhesion of tissue.

What is pulling the tissue from the slide? I am quite confident that *the forces applied to the tissue during agitation* are pulling the tissue from the slide. This can be minimized by dipping the slide gently up and down avoiding swirling motions that put pressure on the face of the slide and edge of the tissue. The perimeter of the tissue is approximately five microns thick and will catch the forces of the agitation. The tissue is being held to the slide by the forces of adhesion over the face of the tissue. The larger the ratio of the perimeter forces pulling off the tissue to the surface area forces holding the tissue to the slide, the easier the tissue will come off the slide. In other words, skinny strips with a large perimeter and little surface area will fall off easier than a rectangular round or square piece of tissue that has more surface holding the tissue to the slide. For this reason, be careful not to over agitate when staining long strips of tissue. Once the edge begins to peel away the forces on the edge increase significantly and it will take little agitation to pull it completely from the slide. Below, I will share some reasons I have found for tissue falling from the slide.

Tissue is very dry by nature or desiccation. Tissues such as very sclerotic fibroconnective tissues are relatively hypovascular and drier by nature. These dry tissues can easily be pulled from the slide with over agitation. Using my analogy it would seem that these tissues have less glue. Tissues such as thin walled ovarian cysts being both sclerotic and having a large perimeter to surface area ratio can easily be shaken from the slide. Tissues which have been allowed to desiccate to the prolonged exposure to air also have a tendency to fall off the slide. I guess that in these tissues our glue has dried out!

Necrotic and liquifactive tissues. Even though we have a large face of tissue holding the section to the slide, there must be something holding the tissue together. Tissues which have become completely necrotic and liquifactive have been reduced to particles of tissue and inflammatory cells no longer having any architectural forces holding the tissue together. An example of this would be a lymph node involved with tumor, in which the central tumor has become totally necrotic. This necrotic tissue can become easily dislodged in the staining process by over agitation.

A section is placed over embedding medium. When picking up multiple sections on a slide, if the tissue form one section is overlapped onto the embedding medium of another section already picked up on the slide, the tissue will not adhere as well to the embedding medium as it will to the glass slide creating a tendency for the second section to fall off the slide.

Ammonia bluing agent too strong. I have had the experience of sections which should adhere nicely, mysteriously falling of the slide in the ammonia water only to find it smelled much stronger than usual. In my experience, if you can smell ammonia without putting your nose to it is more concentrated than it should be.

100% ETOH instead of 95%. I have had the experience of sections mysteriously falling off in what should have been my 95% ETOH only to find that 100% ETOH was placed in the fixative jar.

Formalin fixed tissue. The cohesive nature of the tissue seems to be dramatically reduced after formalin fixation. My only explanation for this would be that the *glue* in tissue fluid responsible for cohesion to the slide are fixed or washed from the tissue in the fixation process.

Frozen sections of bone falling off slides. Keeping frozen sections of bone or cartilage from falling off the slides may be difficult due to the curling affect of cartilaginous tissue. One thing that can be done to keep bone or cartilage on the slide is fixing the slide in Acetone for 1 min prior to putting the slide in 95% alcohol or other fixative.

6.8 Coverslipping

When coverslipping a slide there are a variety of problems we can create which will hinder our interpretation or waste our valuable time. Excessive air bubbles under the coverglass can be created when dropping a cover glass onto the slide. Using too much mounting medium without carefully wiping the edge will cause medium to ooze from beneath the coverslip getting onto the front of the slide and blurring the image. Medium on the back of the slide will gum up the microscope stage making it impossible to drive the slide and require cleaning of the stage. I suggest limiting the mounting medium to a 4 or 5 mm drop. Mounting medium will thicken due to evaporation making it more difficult to spread on the slide and should be changed or diluted with xylene when it becomes unmanageable.

Another common problem is mistakenly putting the coverslip on the back of the slide and then wiping off the tissue from the front. Using slides with only a frosted end without writing and working in busy situations, it is very easy to pick up a slide and retrieve the tissue on the back of the slide. When the slide contains a very tiny piece of tissue, it can be difficult to see which side it is on, especially when it is still wet with xylene. If the tissue was put on the back side, it might go unnoticed until it is wiped away with gauze. When retrieving tissue always take an extra moment to be certain the tissue is going on the front of the slide and take an extra moment to check that the tissue is not on the surface you are about to wipe. It pays to label the slide before retrieving the tissue to make it clear which side is front. A note of caution; beware when prelabeling multiple slides or any container for that matter. If you pick up the wrong labeled slide, you can easily confuse two specimens. This can be a problem particularly in very busy settings handling many specimens at a time. Personally I like to label the slide once I have picked up the

tissue. It will prevent such errors but as a result occasionally I will pick up the tissue on the wrong side of the slide. Especially, when my slide box has been shuffled by the gremlins that frequent so many frozen section rooms. As I have mentioned elsewhere in this book, the best way to avoid mistakes is to develop routines and stick to the at all times. If you do the same thing every time you be less prone to such errors.

There are a variety of ways to apply a coverslip to a slide. Figure 6.5 illustrates a method I have found to work quite quickly and creates few bubbles. Lay out as many coverslips as needed on a flat paper towel about an inch apart. The typical trifold towels work well. Place a drop of embedding medium in the center of each slide. Take the stained frozen section slide still wet with xylene and rest it on the towel along side of the coverslip. Now hinge the slide down 95% of the way to the coverslip so the slide contacts the drop of medium on the coverslip. Do not let it fall all of the way to the coverslip. If the mounting medium begins to spread by capillary action between the two glass surfaces, reverse the slide backward and lie

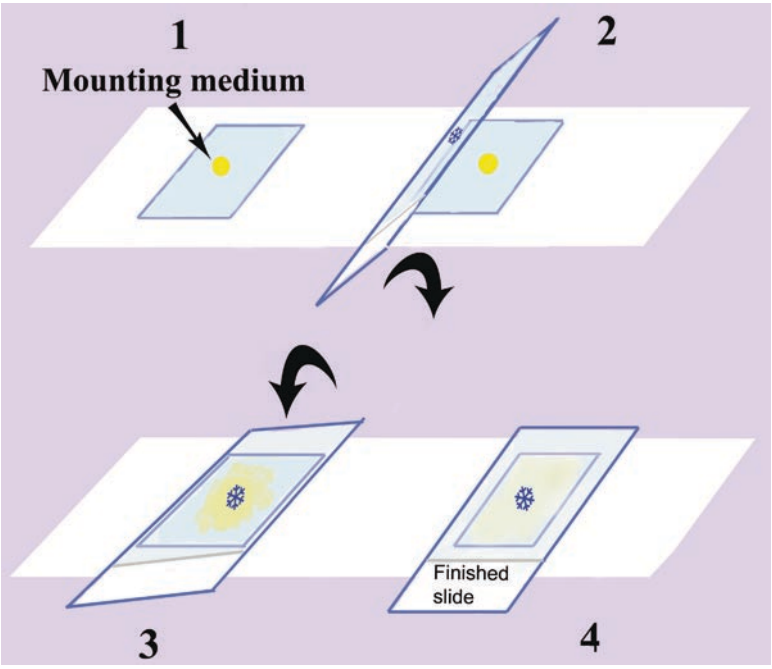

Fig. 6.5 Coverslipping method. Lie a cover slip on a flat paper towel and apply a drop of mounting medium as seen in part 1. Rest the slide on the towel next to the coverslip and hinge it down to the coverslip as in part 2. When the slide engages the drop of medium hinge it backwards as in #3. The medium will spread by capillary action between the glass slide and coverslip. Turn the slide face up on the towel or stand it up against a vertical surface to dry

it face up on the paper towel or stand it up to dry. If done correctly, there will be very few bubbles. If the medium is thick, let a drop of xylene fall from the wetted slide onto the drop of mounting medium to dilute the medium and facilitate rapid capillary action.

6.9 Wiping the Slide

After coverslipping the slide, any excess medium will need to be wiped from the edge of the slide. Skilled histotechnologists develop a fine touch with a piece of gauze allowing them to neatly wipe the edge of the slide. I never quite mastered this method and often found myself catching the edge of the coverglass with this wad of gauze or spreading medium onto the surfaces of slide. I have found a simpler way to wipe my slides that was to tape an absorbent pad or thick paper towel so that it wraps tightly around the edge of a counter. I will tape one side of the towel under the counter next to my microscope. Next, I stretch the towel into the counter and tape in place so that it is taught. This creates a sharp flat towel covered surface on the edge of the bench. Slides can be quickly and easily wiped against this firm flat absorbent surface with little opportunity to catch the coverglass. As the towel gets filled with dried medium, free the towel and move it, so a clean portion overlaps the edge Fig. 6.6.

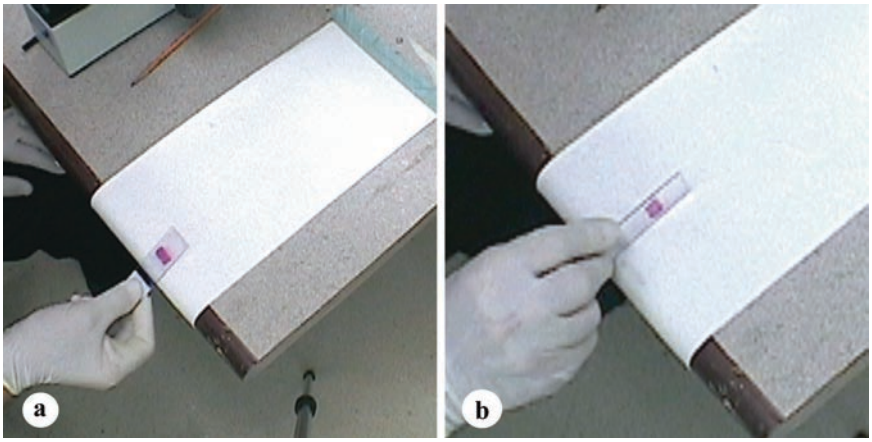

Fig. 6.6 A slide wiping towel stretched and taped over the edge of a counter. The bottom of the slide is being wiped against the towel covered edge of the desk in figure (**a**). The edge of the slide is being wiped in figure (**b**). As the edge of the towel becomes filled with medium the towel can be untapped and moved so a clean area of the towel is available

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