APPENDIX III

Routine Histotechniques, Staining and Notes on Immunohistochemistry

■ S ANIL AND R RAJENDRAN

HISTOTECHNIQUES: TISSUE PROCESSING

Histotechniques constitute the backbone of diagnostic pathology, enabling histologic diagnosis of diseased tissue possible for proper management. The protocol is rigid, custom made based on scientific principles with the aim of avoiding procedural artifacts as far as possible. The exercise is more and more being automated but a good diagnostic slide avoiding the routine artifacts; its make is still an art and skill of a histotechnologist than dictated by any scientific principle. The commonly applied histotechnques, its principle and application in a routine diagnostic laboratory are discussed in this chapter.

SPECIMEN COLLECTION

Tissue specimens received in the surgical pathology laboratory have a request form that lists the patient information and history along with a description of the site of origin. The specimens are accessioned by giving them a number that will identify each specimen for each patient.

GROSS EXAMINATION

Tissues removed from the body for diagnosis arrive in the pathology department and are examined by a pathologist, pathology assistant, or pathology resident. Gross examination consists of describing the specimen and placing all or parts of it into a small plastic cassette which holds the tissue while it is being processed to a paraffin block.

MARGINATING THE GROSS SPECIMEN

When a malignancy is suspected, the specimen is often covered with ink in order to mark the margins of the specimen. Different colored inks can be used to identify different areas if needed. When sections are made and processed, the ink will mark the actual margin on the slide.

FIXATION: TYPES OF FIXATIVES

The purpose of fixation is to preserve tissues permanently in as life-like a state as possible. Fixation should be carried out as soon as possible after removal of the tissues (in the case of surgical pathology) or soon after death (with autopsy) to prevent autolysis. There is no perfect fixative, though formaldehyde comes the closest. Therefore, varieties of fixatives are available for use, depending on the type of tissue present and features to be demonstrated.

There are five major groups of fixatives, classified according to mechanism of action:

- Aldehydes
- Mercurials
- Alcohols
- Oxidizing agents
- Picrates

Aldehydes. These include formaldehyde (formalin) and glutaraldehyde. Tissue is fixed by cross-linkages formed in the proteins, particularly between lysine residues. This cross-linkage does not harm the structure of proteins greatly, so that antigenicity is not lost. Therefore, formaldehyde is good for immunoperoxidase techniques. Formalin penetrates tissues well, but is relatively slow. The standard solution is 10% neutral buffered formalin. A buffer prevents acidity that would promote autolysis and cause precipitation of formol-heme pigment in the tissues.

Glutaraldehyde causes deformation of alpha-helix structure in proteins so is not good for immunoperoxidase staining. However, it fixes very quickly so is good for electron microscopy. It penetrates very poorly, but gives best overall cytoplasmic and nuclear detail. The standard solution is a 2% buffered glutaraldehyde.

Mercurials. These fix tissues by an unknown mechanism. They contain mercuric chloride and include such well-known fixatives as B-5 and Zenker's. These fixatives penetrate

relatively poorly and cause some tissue hardness, but are fast and give excellent nuclear details. Their best application is for fixation of hematopoietic and reticuloendothelial tissues. Since they contain mercury, they must be disposed of carefully.

Alcohols. Including methyl alcohol (methanol) and ethyl alcohol (ethanol), are protein denaturants and are not used routinely for tissues because they cause too much brittleness and hardness. However, they are very good for cytologic smears because they act quickly and give good nuclear detail. Spray cans of alcohol fixatives are marketed to physicians doing PAP smears, but cheap hairsprays do just as well.

Oxidizing Agents. These include permanganate fixatives (potassium permanganate), dichromate fixatives (potassium dichromate), and osmium tetroxide. They cross-link proteins, but cause extensive denaturation. Some of them have specialized applications, but are used very infrequently.

Picrates. These include fixatives with picric acid. Foremost among these is Bouin's solution. It has an unknown mechanism of action. It does almost as well as mercurials with nuclear detail but does not cause as much hardness. Picric acid is an explosion hazard in dry form. As a solution, it stains everything it touches yellow, including skin.

FIXATION – FACTORS AFFECTING FIXATION

There are a number of factors that will affect the fixation process:

- Buffering
- Penetration
- Volume
- Temperature
- Concentration
- Time interval

Fixation is best carried out close to neutral pH, in the range of 6–8. Hypoxia of tissues lowers the pH, so there must be buffering capacity in the fixative to prevent excessive acidity. Acidity favors formation of formalin-heme pigment that appears as black, polarizable deposits in tissue. Common buffers include phosphate, bicarbonate, cacodylate, and veronal. Commercial formalin is buffered with phosphate at a pH of 7.

Penetration of tissues depends upon the diffusibility of each individual fixative, which is a constant. Formalin and alcohol penetrate the best, and glutaraldehyde the worst. Mercurials and others are somewhere in between. One way to get around this problem is sectioning the tissues thinly (2 to 3 mm). Penetration into a thin section will occur more rapidly than for a thick section.

The volume of fixative is important. There should be a 10:1 ratio of fixative to tissue. Obviously, we often get away with less than this, but may not get ideal fixation. One way to partially solve the problem is to change the fixative at intervals to avoid exhaustion of the fixative. Agitation of the specimen in the fixative will also enhance fixation.

Increasing the temperature as with all chemical reactions will increase the speed of fixation, as long as you do not cook the tissue. Hot formalin will fix tissues faster, and this is often the first step on an automated tissue processor. Concentration of fixative should be adjusted down to the lowest level possible, because you will expend less money for the fixative. Formalin is best at 10%; glutaraldehyde is generally made up at 0.25 to 4%. Too high a concentration may adversely affect the tissues and produce artifact similar to excessive heat.

Also very important is time interval from removal of tissues to fixation. The faster you can get the tissue fixed, the better. Artifact will be introduced by drying, so if tissue is left out, keep it moist with saline. The longer you wait, the more cellular organelles will be lost, and the more nuclear shrinkage and artefactual clumping will occur.

FIXATIVES – GENERAL USAGE

There are common usages for fixatives in the pathology laboratory based upon the nature of the fixatives, the type of tissue, and the histologic details to be demonstrated. Formalin is used for all routine surgical pathology and autopsy tissues when an H & E slide is to be produced. Formalin is the most forgiving of all fixatives when conditions are not ideal, and there is no tissue that it will harm significantly. Most clinicians and nurses can understand that formalin smells bad enough so they should be careful when handling it.

Zenker's fixatives are recommended for reticuloendothelial tissues including lymph nodes, spleen, thymus, and bone marrow. Zenker's fixative fixes nuclei very well and gives good detail. However, the mercury deposits must be removed (dezenkerized) before staining or black deposits will result in the sections. Bouin's solution is sometimes recommended for fixation of testis, GI tract, and endocrine tissues. It does not do a bad job on hematopoietic tissues either, and does not require dezenkerizing before staining.

Glutaraldehyde is recommended for fixation of tissues for electron microscopy. The glutaraldehyde must be cold and buffered and not more than 3 months old. The tissue must be as fresh as possible and preferably sectioned within the glutaraldehyde at a thickness not more than 1 mm to enhance fixation.

Alcohols, specifically ethanol, are used primarily for cytologic smears. Ethanol (95%) is fast and cheap. Since smears are only a cell or so thick, there is no great problem from shrinkage, and since smears are not sectioned, there is no problem from induced brittleness.

For fixing frozen sections, you can use just about anything–though methanol and ethanol are the best.

TISSUE PROCESSING

Once the tissue has been fixed, it must be processed to a form in which it can be made into thin microscopic sections. The usual way this is done is with paraffin. Tissues embedded in paraffin, which is similar in density to tissue, can be sectioned at anywhere from 3 to 10 microns, usually 4 – 6 routinely. The technique of getting fixed tissue into paraffin is called tissue sections processing. The main steps in this process are dehydration and clearing.

Wet fixed tissues (in aqueous solutions) cannot be directly infiltrated with paraffin. First, the water from the tissues must be removed by dehydration. This is usually done with a series of alcohols, say 70 to 95% to 100%. Sometimes the first step is a mixture of formalin and alcohol. Other dehydrants can be used, but have major disadvantages. Acetone is very fast, but a fire hazard, so is safe only for small, hand-processed sets of tissues. Dioxane can be used without clearing, but has toxic fumes.

The next step is called 'clearing' and consists of removal of the dehydrant with a substance that will be miscible with the embedding medium (paraffin). The most common clearing agent is xylene. Toluene works well, and is more tolerant of small amounts of water left in the tissues, but is 3 times more expensive than xylene. Chloroform works, but is a health hazard, and is slow. Methyl salicylate is rarely used because it is expensive, but it smells nice (it is oil of wintergreen). There are newer clearing agents available for use. Many of them are based on limolene, a volatile oil found in citrus peels. Others use long chain aliphatic hydrocarbons (Clearite). Although they represent less of a health hazard, they are less compatible with poorly fixed, dehydrated, or sectioned tissues.

Finally, the tissue is infiltrated with the embedding agent, almost always paraffin. Paraffins can be purchased that differ in melting point, for various hardnesses, depending upon the way the histotechnologist likes them and upon the lab conditions (warm vs. cold). A product called paraplast contains added plasticizers that make the paraffin blocks easier for some technicians to cut. A vacuum can be applied inside the tissue processor to assist penetration of the embedding agent. The above processes are almost always automated for the large volumes of routine tissues processed. Automation consists of an instrument that moves the tissues around through the various agents on a predetermined time scale. The 'Technicon' tissue processor is one of the most common and most reliable (a mechanical processor with an electric motor that drives gears and cams), though no longer made. Newer processors have computers, not cam wheels, to control them and have sealed reagent wells to which a vacuum and/or heat can be applied.

TISSUE PROCESSOR

Automated tissue processor

Tissues that come off the tissue processor are still in the cassettes and must be manually put into the blocks by a technician who must pick the tissues out of the cassette and pour molten paraffin over them (Fig. AIII.1). This 'embedding' process is very important, because the tissues must be aligned, or oriented, properly in the block of paraffin.

Tissue Embedding. Alternatives to paraffin embedding include various plastics that allow thinner sections. Such plastics include methyl methacrylate, glycol methacrylate, Araldite, and epon. Methyl methacrylate is very hard and therefore good for embedding undecalcified bone. Glycol methacrylate has the most widespread use since it is the easiest to work with. Araldite is about the same as methacrylate, but requires a more complex embedding process. Epon is routinely used for electron microscopy where very thin sections are required.

Figure AIII.1 Automated tissue processor.

Plastics require special reagents for dehydration and clearing that are expensive. For this reason, and because few tissues are plastic embedded, the processing is usually done by hand. A special microtome is required for sectioning these blocks. Small blocks must be made, so the technique lends itself to small biopsies, such as bone marrow or liver.

SECTIONING

Once the tissues have been embedded, they must be cut into sections that can be placed on a slide. This is done with a microtome. The microtome is nothing more than a knife with a mechanism for advancing a paraffin block standard distances across it.

SECTIONING WITH MICROTOME

Knives are either of the standard thick metal variety or thin disposable variety. The former type allows custom sharpening to one's own satisfaction, but is expensive. The latter costs less and are nearly as good. The advantage of the disposable blade becomes apparent when sectioning a block in which is hidden a metal wire or suture, which damages the cutting edge of the blade. Plastic blocks (methacrylate, Araldite, or epon) are sectioned with glass or diamond knives. A glass knife can section down to about 1 micron. Thin sections for electron microscopy (1/4 micron) are best done with a diamond knife which is very expensive.

Microtomes have a mechanism for advancing the block across the knife. Usually this distance can be set, for most paraffin embedded tissues at 4–6 microns. The more expensive the microtome, the better and longer-lasting this equipment will be. Sectioning tissues is a real art and takes much skill and practice. Histotechnologists are the artists of the laboratory. It

is important to have a properly fixed and embedded block or much artifact can be introduced in the sectioning. Common artifacts include tearing, ripping, 'venetian blinds' holes, folding, etc. Once sections are cut, they are floated on a warm water bath that helps remove wrinkles. Then they are picked up on a glass microscopic slide.

Picking Sections up from Water Bath — Unstained Section on Glass Slide. The glass slides are then placed in a warm oven for about 15 minutes to help the section adhere to the slide. If this heat might harm such things as antigens for immunostaining, then this step can be bypassed and glue-coated slides are used instead to pick up the sections.

HARD TISSUE PROCESSING (BONE, TOOTH)

COMMON FIXATIVES IN HARD-TISSUE PROCESSING

Fixation is one of the most important steps in obtaining a good histological specimen. Its goal is to block all lytic enzyme activity as well as the activity of bacteria and other infectious agents in order to preserve the constituents of a tissue as they were in the living state. The most common solutions employed for fixation include 10-30% formalin, glutaraldehyde, paraformaldehyde, and alcohol-based solutions. Formalin penetrates tissues well, but its action is relatively slow. The standard solution used is 10% neutral buffered formalin (NBF). A buffered solution prevents acidity, which would promote autolysis, causing precipitation of formol-heme pigment in the tissues. The alcohol-based solutions have the advantage of preserving numerous enzymes, allowing the performance of many histochemical studies. All these fixatives are known as primary, and the rest as secondary. The latter are obtained by mixing together several primary fixatives (e.g. Bouin's fixative containing picric acid, formalin, and glacial acetic acid), in order to make use the different advantages presented by each component.

It is also important to reduce the size of the specimen to allow better fixation: the best size of the specimen appears to be 4-5 mm. Although formaldehyde is the best fixative, it is not the perfect one. Therefore, a variety of fixatives are available for use, depending on the type of tissue we want to study and on the features we want to analyze. For specimens with a diameter larger than 4-6 mm, glutaraldehyde and formalin are suggested, while for specimens smaller than 1-3 mm, alcoholic fixatives may be used.

POST-FIXATION TREATMENT AND SPECIMEN STORAGE

The fixed specimen must be washed in phosphate buffered saline (PBS) or running water to completely remove all the fixative solutions, which could affect the staining procedure. Washing in PBS is performed by immersing the specimen for 1 day in PBS, and changing the solution several times. Fixed specimens can be stored in alcohol or fixative for several weeks, but longer periods in alcohol tend to shrink the cells, altering the morphology. For large specimens, water tends to separate from alcohol and the latter also evaporates, leaving a large portion of the tissue under water and without alcohol. Formalin, after long period, tends to lose its fixing capability. If specimens must be preserved for more than several weeks, the best way is to infiltrate and embed them in resin. In this case, there is no time limit for storage.

EMBEDDING TECHNIQUES AND RESULTS IN DIFFERENT TYPES OF RESIN

Plastic-embedding technique generally does not require the removal of the resin before staining, a process that could introduce artifacts in the sections. The presence of the resin in the section makes the staining procedure different from routine paraffin-embedded tissues, and achieving satisfactory staining is more difficult.

An exception is methyl methacrylate (MMA) which is removed from the sections after cutting to permit staining. Embedding in MMA requires the removal of the resin with solvents. Glycol methacrylate cannot be removed because of the high number of crosslinking binding sites present in the chains of the polymer. Sectioning of blocks containing hard endosseous biomaterials (bone implants)can be made easier by the use of a system consisting of a special glycol methacrylate resin (Technovit 7200 VLC, Kulzer, Wehrheim, Germany), which provides good infiltration, polymerization, and subsequent easy sectioning by cutting and grinding the specimens. Although this system allows the precise observation of an intact interface between bone and endosseous biomaterials, the hardness of glycol methacrylate and its permanence in the tissues make any routine staining procedure very difficult.

During the last three decades, the increase in the use of medical and dental implants and techniques of bone regeneration has underlined the importance of the histological evaluation of the tissue-implant interface. Conventional methods of microscopic examination have shown to be inappropriate for studying undecalcified bone, implants, and biomaterials. Thus, various plastic-embedding methods have been used to produce sections in which both the implant material and the adjacent tissues are intact. Many biomaterials cannot be infiltrated with conventional embedding media, and they can be more resistant to grinding than the embedding media and/or the surrounding hard tissues, producing sections of uneven thickness. Plastic embedding allows a distinction between mineralized bone and unmineralized osteoid, with an excellent preservation of the cellular structures.

It must be kept in mind that histological detail is critical for the morphometrical evaluation, diagnosis, and study of the bone pathology. For optimal and reproducible processing of the specimens, the following steps are necessary:

- Optimal procurement of the specimen
- Proper fixation
- Proper embedding
- Proper sectioning
- Proper staining.

Methacrylate-embedding medium is the method of choice for the study of bone. Glycol methacrylate-embedding provides good infiltration of cartilage and good preservation of osteoblast-associated alkaline phosphatase (ALP). Methyl methacrylate (MMA) produces a harder plastic that provides a superior support for cortical and trabecular bone and allows a better preservation than glycol methacrylate of ALP in cartilage matrix and of acid phosphatase (ACP) in osteoclasts. The use of MMA as an embedding medium has made possible the study of semi-thin sections of mineralized bone.

STAGES OF HARD TISSUE HISTOLOGICAL SPECIMEN PREPARATION

Dehydration

Dehydration follows fixation, and it has the goal of removing all the water contained within the specimen to allow uniform penetration of the resin. It also allows the penetration of resin into any biomaterial present. The dehydration process is essential because the resins employed are not water-soluble. The water is removed by immersing the specimen in alcohol solutions of increasing concentration (ascending grades). The time for each step will vary (from 15 minutes to 24 hours), depending on the size of the specimen. The alcohol concentrations used are as follows:

- 30% alcohol
- 50% alcohol
- 70% alcohol
- 90% alcohol
- 100% alcohol
- 100% alcohol.

The time required for dehydration may be reduced when vacuum is applied. The dehydration process hardens the tissues, making resin penetration more difficult. To avoid this problem, a few drops of glacial acetic acid may be added to the 70% alcohol solution. For some resins, acetone dehydration can be performed.

Infiltration

Resins commonly used for infiltration and embedding are:

- Technovit 7200 Kulzer
- Technovit 8100 Kulzer
- Technovit 9100 Kulzer
- Epon
- L-R White
- Other MMA resins
- Spurr's resin.

All these resins are initially fluids, and solidify during polymerization. Resin embedding produces hard blocks containing the tissues to be trimmed and cut for examination. The resins must be put in appropriate embedding molds that do not react with the resin.

Embedding and Polymerization

After embedding, polymerization is performed for about 6-8 hours at a temperature not exceeding 40°C. The polymerized resin is very hard, and it is possible to cut and grind the specimen in a very uniform way with no alteration of the histological features. Polymerization may be accomplished either by using light polymerization or autopolymerization, depending on the type of resin used. Once the block is obtained, for light microscopy observation, it can be glued to either a base of Plexiglas or a clean glass slide. Several types of glue are suitable (e.g. Technovit 4000, Attack, Vitroresin). The surface to be examined must be positioned uppermost. Excess resin is removed in order to obtain a free surface of the specimen parallel to the holding slide. At this stage another slide is glued to the free surface. However, the most superficial portion of the resin usually remains fluid as the presence of oxygen in the air inhibits the polymerization process. This excess resin is removed with a knife or by grinding and the clean glass slide can be attached with glue.

Block Trimming

The polymerized blocks must be removed from their molds. Before trimming the plastic block, the orientation of the specimen in the block to the microtome blade has to be done. The cylindrical block containing a bone/implant core is flattened on a plane parallel to the length of the core. Flattening can be done on a grinder/polisher machine with coarse silicon carbide paper. The grinding paper should be flushed with a stream of water to prevent plastic particles from clogging the paper. Leave at least 1 mm of plastic between each cut and the bone specimen. To remove the excess plastic on the sides of the specimen, two more cuts are made, each perpendicular to the end of the first two cuts. The shape and preparation of the base of the block is done according to the microtome that is used for slicing.

Several techniques have been reported that describe the preparation of non demineralized bone specimens for study at both the light and electron microscopic levels.

Microtome Sectioning

Types of microtomes for sectioning bone

Microtomes generally produce two movements: the **cutting movement** of the knife along the desired plane, and the **feeding movement** of the specimen block perpendicular to the cutting plane. Microtomes of good quality have precise adjustable movements. Most microtomes from well-known manufacturers meet these demands. Large, be sectioned with a sliding or base sledge microtome, where in either case the specimen block remains stationary while the knife is passed through it. The knife is fastened in a holder, which slides back and forth on rails with the cutting edge of the blade in the lead. The specimen is advanced in small increments at a right angle into the path of the oncoming knife-edge. As the knife sweeps past the specimen, a thin slice or section is

made. The uniform thickness and flatness of the section is determined by the sharpness of the blade, the stoutness of the cutting-edge, the steadiness of the knife motion, and the ability of the apparatus to absorb vibration. For this purpose, these microtomes are stable and robust, and are motorized to provide a slow and steady cutting motion.

Saw microtome (horizontal diamond saw)

A horizontal rotation-sawing machine with diamond cutting edge on the inside diameter is used for sectioning of the dental implant/bone interface studies. The inside diameter diamond saw blade consists of a thin circular steel core, which is tensioned at its outer edge in two clamping rings. A rigid and vibration-free assembly is required to obtain exact thickness of the sections. A powerful and silent motor drives the saw. Samples of bone containing implanted biomaterials are embedded in methyl methacrylate. After polymerization, the blocks are trimmed and firmly fixed to a flattened ball. The ball is tightly clamped into an arm-type holder that moves toward the diamond saw with a force of 0.2-2 Nm, using a 1:1 (v/v) glycerin/water mixture as a cooling lubricant. The block is fixed in the holder at a defined angle (free motions of 360° horizontally and 60° vertically). Thick sections are cut until the desired area of the specimen is reached, then the surface is etched for 30seconds with 1% ethanol-HCl solution and rinsed with water. The surface of the sample is stained with methylene blue (1 minute) and basic fuchsin blue (30 seconds). After staining, the surface is rinsed with water and is carefully dried, then a glass cover slip is glued to the stained surface with a thin layer of UV adhesive; this coverslip stabilizes the thin section during the sectioning process. The block is raised using a high-quality micromanipulator (Mikrocontrol UT 100, Elmekanic®, Markelo, The Netherlands) with a reliable read-off system for a precise sample lift of 1 mm, enabling production of sections of exact thickness. After sawing, the previously stained section with cover slip attached is glued to a glass slide with Permacol and is ready for histological evaluation.

Staining

Toluidine blue, acid fuchsin, silver nitrate, acid and alkaline phosphatase are routinely used staining agents. Acid solutions may alter the resin properties, and can produce a background staining that does not allow an accurate morphologic evaluation. Deplasticized stained sections on glass slides are mounted in the same way as paraffin sections using alcohol dehydration, xylene clearing, and mounting with a synthetic mounting medium. Free floating sections tend to wrinkle, and while in clearing agent, can be flattened with a brush or rolled between pieces of smooth filter paper, then mounted with synthetic resin with a weight placed on top of cover glass to keep the section flat until the medium dries. Clamping devices maintain flat sections briefly but are too strong, causing the mounting media to retract later during storage. Sections cleared with terpineol and mounted with terpene based mounting media may be flatter initially, but this medium doesnot harden as well as the synthetic resins and results in an unstable mount.

Surface-stained sections surrounded by MMA cannot be dehydrated, cleared or mounted. Methylmethacrylate is softened by alcohols and is soluble in xylene and the solvents in mounting media, and any mounting results in ugly cracking of plastic in and around a section. To examine surface-stained sections, place a cover glass on top of the dry section without mounting media and examine with the brightest light setting on the microscope. Immersion oil can be used to mount these sections, but is temporary and leaves messy oil residue on stored sections.

Hematoxylin and Eosin for MMA-embedded Tissue

This method is useful for diagnosis of suspected osteomalacia and distinguishes mineralized bone from osteoid, with nuclei and other soft tissues stained similar to decalcified bone-paraffin sections.

HEMATOXYLIN AND EOSIN

Reagents

Cole's hematoxylin and 1% aqueous eosin.

Method

1. Deplasticize with xylene and hydrate sections to distilled water.
2. Stain in freshly filtered Cole's hematoxylin, 60 minutes. with occasional agitation.
3. Wash well in alkaline tap water.
4. Stain in eosin solution, 30 minutes.
5. Wash in tap water.
6. Dehydrate, clear and mount.

Results

Osteoid -*pink*
Calcified bone-*purplish brown*
Nuclei-*blue.*

Solochrome Cyanine Method

The solochrome cyanine stain differentiates osteoid from newly laid-down bone and older bone.

Solutions

- *Stain solution*
- Solochrome cyanine R - 1 g
- Concentrated sulfuric acid - 2.5 ml.

Mix well until dye incorporates into the resulting 'sludge', then add 500 ml of 0.5% aqueous iron alum (ferric ammonium sulfate), mix and filter.

Method

- Deplasticize with xylene and hydrate sections to distilled water.
- Stain in solochrome cyanine solution, 60 minutes.
- Using a microscope, differentiate in warm (30°C) alkaline tap water until mineralized areas appear blue and other areas light red. Over-differentiation causes all parts to become blue.
- Dehydrate, clear and mount.

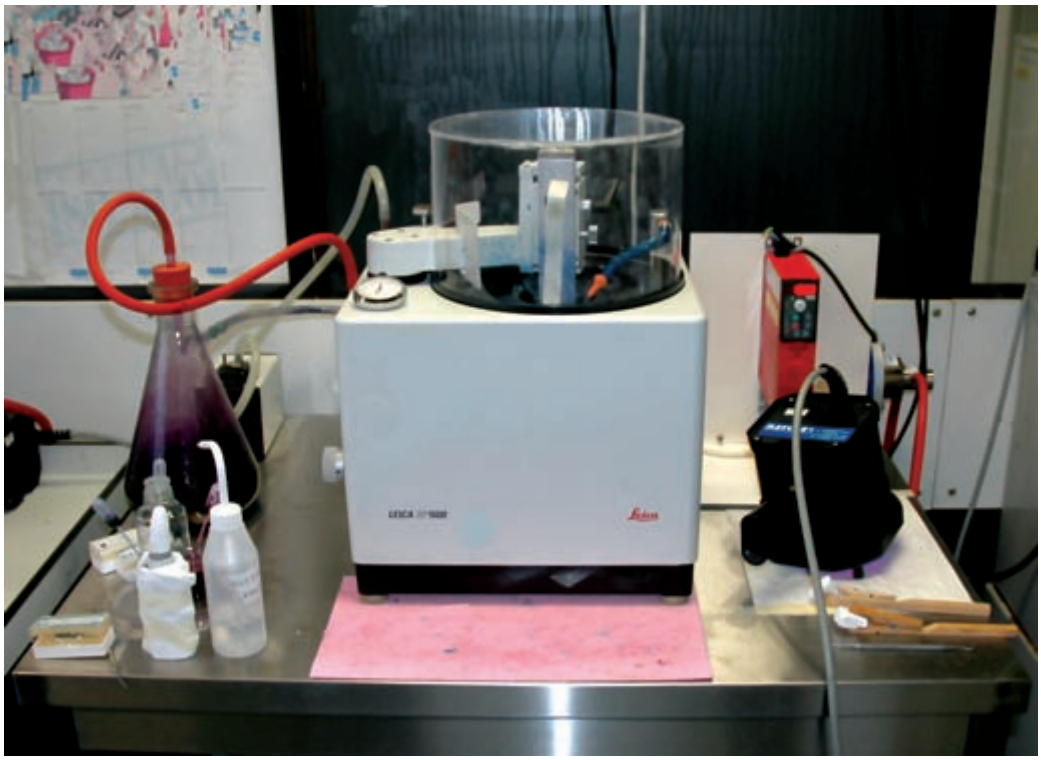


Figure AIII. The Leica SP1600 microtome uses a diamond-coated inner-hole saw blade.

Results

Mineralized bone -*light blue*
Calcification front-*dark blue*
Osteoid-*light red-orange*
Wide osteoid-*light red-orange with pale blue and orange bands*
Nuclei-*blue.*

Staining for Bone Mineral

The classic von Kossa silver method is used to stain the mineral component in bone, (calcium phosphate) and basically is a negative stain for osteoid with the calcium component blackened by silver deposition. Osteoid is counterstained red by either van Gieson's or safronin O. This can also be used as a ground section surface stain but without the acid 'etching' removal of calcium. An alternative method is the rapid bone stain with van Gieson.

A Modification of Von Kossa's Method

Solutions

- 1 % aqueous silver nitrate
- 2.5% sodium thiosulfate
- 1% safranin O or van Gieson's picro fuchsin.

Method

- Deplasticize with xylene, and hydrate sections to distilled water.
- Place in silver nitrate solution, expose to strong light 10-60 min., and watch the mineralized bone turn dark brown to black, indicating a completed reaction.
- Wash in three changes of distilled water.
- Treat with sodium thiosulfate, 5 minutes.
- Wash well in distilled water.
- Counterstain as desired.
- Dehydrate, clear and mount.

Results

- Mineralized bone-*black*
- Osteoid- *red.*

Long wavelength UV light from sunlight or a quartz halogen microscope lamp is preferable to a tungsten filament light bulb, and accelerates the reaction. van Gieson's picro fuchsin counterstaining may interfere with birefringence of osteoid.

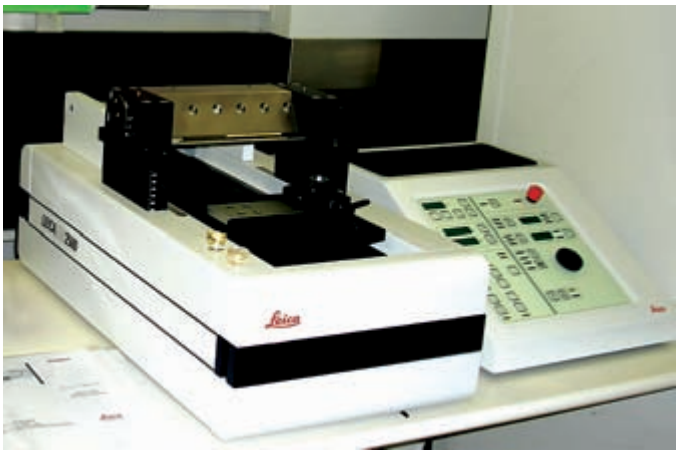
Goldner's Trichrome Method

This staining technique can be more valuable than the von Kossa method in investigations of metabolic diseases, i.e. Paget's, renal osteodystrophy and hyperparathyroidism, because of excellent staining of cells. Osteoblast and osteoclast activity is easily assessed, an important factor for both diagnosis and evaluating the effects of treatment in these disorders by repeated bone biopsies. An additional advantage is that metastatic tumor cells in bone marrow are easily identified.

Solutions

- *Weiffert's iron hematoxylin*
- *Ponceau-fuchsin-azophloxin stock solutions*
- Ponceau de xylidine-0.75 g

A



B

Figure. AIII.2. A, Rotary microtome, B, Sliding microtome.

- Acid fuchsin-0.25 g
- Acetic acid-1.0 ml mix, and add to 100 ml distilled water.
- Azophloxin-0.5 g
- Acetic acid-0.6 ml
- Mix, and add to 100 ml distilled water.

Final working stain solution

- Ponceau-fuchsin solution - 5-10 ml
- Azophloxin - 2 ml
- 0.2% Acetic acid solution - 88 ml

Light green solution

Light green - 1 g
Acetic acid - 1 ml
Mix, and add to 500 ml distilled water

Phosphomolybdic acid/orange G solution

Phosphomolybdic acid - 3 g
Orange G - 2 g
Dissolve in 500 ml of distilled water, and add a crystal of thymol.

Method

- Deplasticize with xylene and hydrate sections to water.
- Immerse sections in alkaline alcohol solution (90 ml of 80% ethanol and 10 ml of 25% ammonia), 1 hour.
- Rinse in water, 15 minutes.
- Stain in Weigert's hematoxylin, I hour.
- Rinse in tap water, 10 minutes.
- Rinse in distilled water, 5 minutes.
- Stain in final Ponceau-fuchsin- azophloxin solution, 5 minutes.
- Rinse in 1% acetic acid, 15 seconds.
- Stain in phosphomolybdic acid/orange G solution, 20 minutes.
- Rinse in 1% acetic acid, 15 seconds.
- Stain with light green, 5 minutes.
- Rinse in three changes of 1% acetic acid.
- Rinse in distilled water, blot dry and mount.

Results

Mineralized bone-*green*, Osteoid-*orange/red*, Nuclei-*blue-gray*, Cartilage-*purple.*

An YH, Martin KL. Handbook of histology methods for bone and cartilage: Humana Pr Inc; 2003.

Frozen Sections. At times during performing surgical procedures, it is necessary to get rapid diagnosis of a pathologic process. The surgeon may want to know if the margins of his/her resection for a malignant neoplasm are clear from tumor before closing, or an unexpected disease process may be found and require diagnosis to decide what to do next, or it may be necessary to determine if the appropriate tissue has been obtained for further work-up of a disease process. This is accomplished through use of a frozen section. The piece(s) of tissue to be studied are snap frozen in a cold liquid or cold environment (-20 to -70 Celsius). Freezing makes the tissue solid enough to section with a microtome.

Frozen sections are performed with an instrument called a cryostat. The cryostat is just a refrigerated box containing a microtome. The temperature inside the cryostat is about -20 to -30 Celsius. The tissue sections are cut and picked up on a glass slide. The sections are then ready for staining.

STAINING

The embedding process must be reversed in order to get the paraffin wax out of the tissue and allow water soluble dyes to penetrate the sections. Therefore, before any staining can be done, the slides are 'deparaffinized' by running them through xylenes (or substitutes) to alcohols to water. There are no stains that can be done on tissues containing paraffin.

The staining process makes use of a variety of dyes that have been chosen for their ability to stain various cellular

Fig. AIII.3. Tissue floatation bath.

Fig. AIII.4. Freezing microtome (cryostat for sectioning frozen tissue).

components of tissue. The routine stain is that of hematoxylin and eosin (H and E). Other stains are referred to as 'special stains' because they are employed in specific situations according to the diagnostic need.

Using Automated Stainer. Frozen sections are stained by hand, because this is faster for one or a few individual sections. The stain is a 'progressive' stain in which the section is left in contact with the stain until the desired tint is achieved.

Fig. AIII.5. Automated linear slide stainer.

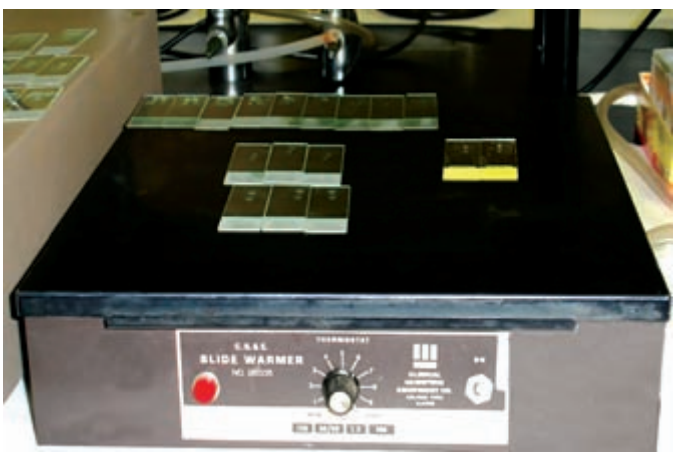


Fig. AIII.6. Slide warmer.

Staining a Frozen Section

H and E Staining (Table AIII.1). Hematoxylin is the oxidized product of the logwood tree (*Haematoxylon campechianum*) known as hematein. Since this tree is very rare nowadays, most hematein is of the synthetic variety. In order to use it as a stain it must be 'ripened' or oxidized. This can be done naturally by putting the hematein solution on the shelf and waiting several months, or by buying commercially ripened hematoxylin or by putting ripening agents in the hematein solution.

Hematoxylin will not directly stain tissues, but needs a 'mordant' or link to the tissues. This is provided by a metal cation such as iron, aluminum, or tungsten. The variety of hematoxylins available for use is based partially on choice of metal ion used. They vary in intensity or hue. Hematoxylin, being a basic dye, has an affinity for the nucleic acids of the cell nucleus.

Hematoxylin stains are either 'regressive' or 'progressive'. With a regressive stain, the slides are left in the solution for a set period of time and then taken back through a solution such

Table AIII.1. H and E Staining (Paraffin)

Hydrate	
1. Xylene	5 minutes
2. Xylene	5 minutes
3. Xylene	5 minutes
4. 100% EtOH	1 minute
5. 100% EtOH	1 minute
6. 95% EtOH	1 minute
7. 95% EtOH	1 minute
8. 70% EtOH	1 minute
9. Deionized water	2 minutes
Stain	
1. Hematoxylin (Gill's 2x)	2 minutes (Filter when metallic scum appears on surface)
2. Tap water rinse under running	
water until water is clear	
3. Bluing solution	1–2 dips
4. Allow slides to stand in running	
tap water for 5 minutes	
(don't use ice-cold water)	
5. 80% EtOH	2 minutes
6. Eosin	2-3 minutes
Dehydrate	
1. 80% EtOH	3–4 dips to rinse out excess Eosin
2. 95% EtOH	1 minute
3. 95% EtOH	1 minute
4. 100% EtOH	1 minute
5. 100% EtOH	1 minute
6. Xylene	3 minutes
7. Xylene	3 minutes
8. Xylene	3 minutes

W Coverslip with Permount (Xylene based). After coverslipping, the chromatin in nuclei should be stained dark blue (hematoxylin) and the cytoplasm should be stained pink (eosin)
EtOH – Ethyl alcohol

as acid-alcohol that removes part of the stain. This method works best for large batches of slides to be stained and is more predictable on a day to day basis. With a progressive stain the slide is dipped in the hematoxylin until the desired intensity of staining is achieved, such as with a frozen section. This is simple for a single slide, but lends itself poorly to batch processing.

Eosin is an acidic dye with an affinity for cytoplasmic components of the cell. There are a variety of eosins that can be synthesized for use, varying in their hue, but they all work about the same. Eosin is much more user friendly than hematoxylin and is less of a problem in the lab. The only problem you will see is overstaining, especially with decalcified tissues.

Coverslipping. The stained section on the slide must be covered with a thin piece of plastic or glass to protect the tissue from being scratched, to provide better optical quality for viewing under the microscope, and to preserve the tissue section for years to come. The stained slide must go through the reverse process that it went through from paraffin section to water. The stained slide is taken through a series of alcohol solutions to remove the water, then through clearing agents to a point at which a permanent resinous substance beneath the glass coverslip or a plastic film can be placed over the section.

Decalcification. Some tissues contain calcium deposits which are extremely firm and which will not section properly with paraffin embedding owing to the difference in densities between calcium and paraffin. Bone specimens are the most likely type here, but other tissues may contain calcified areas as well. This calcium must be removed prior to embedding to allow sectioning. A variety of agents or techniques have been used to decalcify tissue and none of them work perfectly. Mineral acids, organic acids, ethylene diamine tetra acetic acid (EDTA), and electrolysis have all been used.

Strong mineral acids such as nitric and hydrochloric acids are used with dense cortical bone because they will remove large quantities of calcium at a rapid rate. Unfortunately, these strong acids also damage cellular morphology, so are not recommended for delicate tissues such as bone marrow. Organic acids such as acetic and formic acid are better suited to bone marrow, since they are not as harsh. However, they act more slowly on dense cortical bone. Formic acid in a 10% concentration is the best all-around decalcifier. Some commercial solutions are available that combine formic acid with formalin to fix and decalcify tissues at the same time. EDTA can remove calcium and is not harsh (it is not an acid) but it penetrates tissue poorly and works slowly and is expensive in large amounts. Electrolysis has been tried in experimental situations where calcium had to be removed with the least tissue damage. It is slow and not suited for routine daily use.

ARTIFACTS IN HISTOLOGIC SECTIONS AND TROUBLESHOOTING

A number of artifacts that appear in stained slides may result from improper fixation, from the type of fixative, from poor dehydration and paraffin infiltration, improper reagents, and poor microtome sectioning. The presence of a fine black precipitate on the slides, often with no relationship to the tissue (i.e. the precipitate appears adjacent to tissues or within interstices or vessels) suggests formalin-heme pigment has formed. This can be confirmed by polarized light microscopy, because this pigment will polarize a bright white (and the slide will look like many stars in the sky). Formalin-heme pigment is most often seen in very cellular or bloody tissues, or in autopsy tissues, because this pigment forms when the formalin buffer is exhausted and the tissue becomes acidic, promoting the formation of a complex of heme (from red blood cells) and formalin. Tissues such as spleen and lymph node are particularly prone to this artifact. Making thin sections and using enough neutral-buffered formalin (10 to 1 ratio of fixative to tissue) will help. If the fixative solution in which the tissues are immersed is grossly murky brown to red, then place the tissues in new fixative.

The presence of large irregular clumps of black precipitate on slides of tissues fixed in a mercurial fixative such as B-5 suggests that the tissues were not dezenkerized prior to staining. These black precipitates will also appear white with polarized light microscopy. Tissues that are insufficiently dehydrated prior to clearing and infiltration with paraffin wax will be hard to section on the microtome, with tearing artifacts and holes in the sections. Tissue processor cycles should allow sufficient time for dehydration, and final ethanol dehydrant solution should be at 100% concentration. In humid climates, this is difficult to achieve. Covering or sealing the solutions from ambient air will help. Air conditioning (with refrigerants, not with evaporative coolers) will also reduce humidity in the laboratory. Toluene as a clearing agent is more forgiving of poorly dehydrated tissues, but it is more expensive and presents more of a health hazard than other non-xylene clearing agents. Though alcohols such as ethanol make excellent fixatives for cytologic smears, they tend to make tissue sections brittle, resulting in microtome sectioning artifacts with chattering and a 'venetian blind' appearance. Bubbles under the coverslip may form when the mounting media is too thin, and as it dries air is sucked in under the coverslip. Contamination of clearing agents or coverslipping media may also produce a bubbled appearance under the microscope.

PROBLEMS IN TISSUE PROCESSING

are small pieces of tissue that appear on a slide that do not belong to it. They have floated in during processing. Floaters may arise from sloppy procedure on the cutting bench –dirty towels, instruments, or gloves can have tissue that is carried over to the next case. Therefore, it is essential that you do only one specimen at a time and clean thoroughly before opening the container of the next case. The best way to guard against unrecognized floaters is separate similar specimens in the numbering sequence. For example, if you have three cases with prostate chips, separate them in accessioning with totally different specimens such as uterus or stomach. That way, if numbers are transposed or labels written wrong or tissue carried over, then you will have an obvious mismatch. Carrying over one prostate to another, or transposing the numbers of identical tissues may never be recognized.

If reusable cassettes are employed, you must be aware that tissue may potentially be carried over and appear as 'floaters' even several days later, when the cassette is re-used. The problem arises when, during embedding, not all the tissue is removed from the cassette. Then, in the cleaning process, not all of the wax is removed. Then, the next person using the cassette does not pay attention to the fact that there is tissue already in the cassette and puts his/her specimen in it. The floater that appears on the slide will look well-preserved–it should, because it was processed to paraffin. Always be sure that you properly identify the tissue! This means that you make sure that the patient label on the specimen container matches that of the request slip. An accession number is given to the specimen. This number must appear with the tissue at all times. You must never submit a cassette of tissue without a label. You must never submit a cassette of tissue with the wrong label. Mislabeling or unlabeling of tissues is courting disaster.

SAFETY IN THE LAB

The laboratory should be well-ventilated. There are regulations governing formalin and hydrocarbons such as xylene and toluene. There are limits set by the Occupational Safety and Health Administration (OSHA) that should not be exceeded. These limits have recently been revised to reduced levels. Every chemical compound used in the laboratory should have a materials safety data sheet on file that specifies the nature, toxicity, and safety precautions to be taken when handling the compound. The laboratory must have a method for disposal of hazardous wastes. Healthcare facilities processing tissues often contract this to a waste management company. Tissues that are collected should be stored in formalin and may be disposed by incineration or by putting them through a 'tissue grinder' attached to a large sink (similar to a large garbage disposal unit).

Every instrument used in the laboratory should meet electrical safety specifications and have written instructions regarding its use. Flammable materials may only be stored in approved rooms and only in storage cabinets that are designed for this purpose.

Fire safety procedures are to be posted. Safety equipment including fire extinguishers, fire blankets, and fire alarms should be within easy access. A shower and eyewash should be readily available. Laboratory accidents must be documented and investigated with incident reports and industrial accident reports.

Specific hazards that you should know about include:

- Bouin's solution is made with picric acid. This acid is only sold in the aqueous state. When it dries out, it becomes explosive.
- Many reagent kits have sodium azide as a preservative. You are supposed to flush solutions containing sodium azide down the drain with lots of water, or there is a tendency for the azide to form metal azides in the plumbing. These are also explosive.
- Benzidine, benzene, anthracene, and naphthol containing compounds are carcinogens and should not be used.
- Mercury-containing solutions (Zenker's or B-5) should always be discarded into proper containers. Mercury, if poured down a drain, will form amalgams with the metal that build up and cannot be removed.

DIAGNOSTIC CYTOLOGY

Exfoliative cytology has been used as a diagnostic test for precancerous and cancerous lesions presenting in the oral cavity. George N Papanicolaou introduced cytology as a tool to detect cancer and precancer in 1928. It is now a widely accepted method for mass screening in asymptomatic population. However, in general, cytology has relied primarily on the personal judgement of the cytologist and not on

the measurement of cellular parameters. Controversy has surrounded the use of cytology in the diagnosis of oral cancer. One of the main reasons for this is the occurrence of false negative results. A number of authors have suggested reasons for this which includes inadequate sampling, technical errors, misinterpretation of the results and observer bias. It is important that techniques are developed to aid in the diagnosis of early oral cancer especially in predicting the behavior of those lesions which display epithelial dysplasia but no overt malignancy (precancer). In 1981, Cowper and Longmore felt that the application of quantitative techniques to cytology could markedly improve its diagnostic sensitivity in detecting oral cancer. The quantitative techniques included DNA cytophotometry and cytomorphology (measurement of nuclear size, and later cell size).

The major value of cytology is the non-invasive nature of a simple and relatively painfree procedure which can provide intact cells from different layers within the epithelium. Cytology has been recommended for the early diagnosis of oral cancer and proved to be a reliable diagnostic test.

FIXATION OF CYTOLOGY SPECIMENS

Rapid fixation of smears is necessary to preserve cytologic details of cells spread on a glass slide. Fixation means prevention of degeneration of cells and tissue by the autolytic enzymes present in the cells and preservation of cells as close as possible to the living state. To achieve this, smears are placed in the fixative solution for specific periods of time before the staining procedure is started. Fixation changes the physical and chemical state of the cell and determines the subsequent staining reactions that could be carried out on the smears.

PROPERTIES OF CYTOLOGIC FIXATIVES

- Do not excessively shrink or swell cells.
- Do not distort or dissolve cellular components.
- Inactivate enzymes and preserve nuclear details.
- Kill microbes.
- Improve optical differentiation and enhance staining
- Properties of the tissues and cell components are preserved.

CYTOLOGIC FIXATIVES

Wet Fixation

A. Routine Fixatives. The process of immersing freshly prepared smears immediately in a liquid fixative is called wet fixation. This is the ideal method for fixing all gynecological and non-gynecological smears, and any of the following alcohols can be used. All alcohol fixatives should be discarded or filtered (Whatman No: I filter paper) after each use.

- **95% Ethyl alcohol (Ethanol).** The ideal fixative recommended in most of the laboratories for cytological specimen is 95% ethanol alone. It produces the characteristic desired effect on nucleus. It is a dehydrating agent and causes cell shrinkage as it replaces water. But it causes only the desired amount of cell contraction to yield optimal chromatin detail characteristic of cytological preparations. Absolute (100%) ethanol produces a similar effect on cells, but is much more expensive.
- **Ether- alcohol mixture.** This fixative was originally recommended by Papanicolaou. It consists of equal parts of ether and 95% ethyl alcohol. It is an excellent fixative, but ether is not used in most of the laboratories because of its safety hazards, odor and hygroscopic nature.
- **100% Methanol.** 100% methanol is an acceptable substitute for 95% ethanol. Methanol produces less shrinkage than ethanol, but it is more expensive than ethanol.
- **80% Propanol and Isopropanol.** Propanol and isopropanol cause slightly more cell shrinkage than ether-ethanol or methanol. By using lower percentage of these alcohols, the shrinkage is balanced by the swelling effect of water on cells. Hence 80% propanol is a substitute for 95% ethanol.
- **Denatured alcohol.** It is ethanol that has been changed by the additives in order to render it unsuitable for human consumption. There are many different formulae for denatured alcohol; all of them contain methanol as the main ingredient, and hence this can be used at concentrations of 95% or 100%. One formula is 90 parts of 95% ethanol + 5 parts of 100% methanol + 5 parts of 100% isopropanol.

Time of Fixation. Minimum 15 minutes fixation prior to staining is essential. Prolonged fixation for several days or even few weeks will not affect the morphology of cells. If smears are to be preserved over a long period of time in alcohol, it is better to store them in capped containers in the refrigerator.

B. Coating Fixative. Coating fixatives are substitutes for wet fixatives. They are either aerosols applied by spraying the cellular samples or a liquid base, which is dropped onto the slide. They are composed of an alcohol base, which fixes the cells and wax like substance, which forms a thin protective coating over the cells, e.g. Carbowax (polyethylene glycol) fixative. Diaphine fixative spray is a coating fixative (hairspray) with high alcohol content and a minimum of lanolin or oil is also an effective fixative.

Most of these agents have a dual action in that they fix the cells and, when dry, form a thin protective coating over the smear. These fixatives have practical value in situations where smears have to be mailed to a distant cytology laboratory for evaluation. This method is not recommended for smears prepared from fluid within the laboratory as in any good method of fixation the coating fixative should be applied immediately on fresh smears. The distance from which the slides are sprayed with an aerosol fixative affects the cytology details. 10 to 12 inches (25–30 cm) is the optimum distance recommended for aerosol fixative. Aerosol sprays are not recommended for bloody smears, because they cause clumping of erythrocytes. Waxes and oils from hairspray fixative alter staining reactions if they are not adequately removed. Prior to staining, the slides have to be kept overnight in 95% alcohol for removal of the coating fixative.

C. Special Purpose Fixative

Carnoy's fixative. This is a special purpose fixative for hematologic samples. The acetic acid in the fixative hemolyses the red blood cells. It is an excellent nuclear fixative as well as preservative for glycogen but results in considerable shrinkage of cells and tends to produce overstaining with hematoxylin. Overfixing in Carnoy's fixative also results in loss of chromatin material. Carnoy's fixative must be prepared fresh when needed and discarded after each use. It loses its effectiveness on long standing, and chloroform can react with acetic acid to form hydrochloric acid.

AAF Fixative. This is the ideal fixative used for cellblock preparation of fluid specimens.

Mailing of Unstained Smears – Glycerine Method for Mailing Slides. Smears are first fixed in 95% ethanol for 12 minutes. Two drops of glycerine are placed on smears and covered with a clean glass slide. This may be wrapped in wax paper and mailed to the laboratory in a suitable container. Coating fixative such as carbowax fixative and spray coating fixative can be used primarily to facilitate transport of smears, mailing, etc.

Prefixation of Cytologic Material. Prefixation may preserve some specimens for days without deterioration of cells. Some of the disadvantages of prefixation are precipitation/coagulation of proteins, hardening of cells into spherical shapes and condensation of chromatin. The coagulation of proteins may interfere with the adherence of cells to glass slides. It also 'rounds up' the cells – causes the cells to clump together into tight clusters making stain absorption and interpretation difficult. Albuminized slides should be used to prepare smears from prefixed sample. The most common solutions used for this purpose are:

- Ethyl alcohol (50% solution)
- Saccomanno's fixative (50% alcohol with 2% Carbowax 1540)
- Mucolexx (A commercial mucoliquefying preservative for the collection of mucoid and fluid specimens)

Many other preservatives have been developed for use with automated cytology systems.

Rehydration of Air-dried Smears. Unfixed, air-dried gynecological smears received from peripheral areas can be used for Papanicolaou staining by rehydration method. The simplest rehydration technique is to place air- dried cytological specimens in 50% aqueous solution of glycerine for three minutes followed by two rinses in 95% ethyl alcohol, and then stained by the routine Papanicolaou method.

STAINING METHODS IN CYTOLOGY

PAPANICOLAOU STAINING METHOD

Papanicolaou staining method is the routine staining procedure used in cytopathology laboratory. This technique is named after Dr George N Papanicolaou, the father of exfoliative cytology and is devised for the optimal visualization of cells exfoliated from epithelial surfaces of the body. It is a polychrome staining reaction designed to display the many variations of cellular morphology showing degrees of cellular maturity and metabolic activity. The use of the Papanicolaou stain results in well- stained nuclear chromatin, differential cytoplasmic counterstaining and cytoplasmic transparency.

Steps of Staining Procedure (Table AIII.2)

1. **Fixation.** The cytology smears are fixed in 95% ethyl alcohol or in other substitutes for a minimum of 15 minutes.
2. **Nuclear Staining.** It is done by using hematoxylin stain. Harris hematoxylin or its modified form is used in Papanicolaou staining in regressive method, in which we deliberately overstain with hematoxylin and remove the excess stain by using a differentiating solution such as acid alcohol (0.05% HCl in 70% ethyl alcohol) or 0.05%

Table AIII.2. Papanicolaou staining procedure

1. 90% Ethanol (fixation)	15 minutes(mt)
2. 80% Ethanol	2
3. 60% Ethanol	2
4. Distilled water	5 dips
5. Distilled water	5 dips
6. Hematoxylin stain	2.
7. 0.05% HCl solution	2
8. Running tap water (Bluing)	10
9. 60 % Ethanol	2
10. 80% Ethanol	2
11. 80% Ethanol	2
12. 95% Ethanol	2
13. OG-6 stain	2
14. 95% Ethanol	2
15. 95% Ethanol	2
16. 95% Ethanol	2
17. EA-36 Stain	2
18. 95% Ethanol	2
19. 95% Ethanol	2
20. 95%Ethanol	2
21. 95% Ethanol	2
22. Absolute Ethanol	2
23. Absolute Ethanol	2
24. Absolute Ethanol	2
25. Absolute Ethanol+ Xylene (1:1)	2
26. Xylene	5
27. Xylene	5
28. Xylene	till clear
29. Mounting in DPX	

aqueous solution of HCl alone. As hematoxylin is used in an acid pH, a pink color will form and it is not stable. In order to make it stable, the compound is brought to alkaline pH (bluing) by treating with a weak alkaline solution. Running tap water which is slightly alkaline (pH 8) is used as bluing solution in small laboratories. Ammonium hydroxide solution (15 ml of ammonium hydroxide 28 – 30% weight/volume to 985 ml of 70% ethanol) can also be used.

3. **Cytoplasmic Staining.** Cytoplasmic stains are OG-6 and EA-36. Both are synthetic stains and OG-6 is a monochrome stain while EA-36 is a polychrome stain.
4. **Dehydration.** Rinse the smears in absolute alcohol for two or three changes for the removal of water. Smears left in rinses for long will lose too much stain. Alternative to 100% ethanol are 100% isopropanol and 100% denatured alcohol. Rectified spirit affects the cytoplasmic staining and hence is not recommended.
5. **Clearing.** Cells are not transparent while the smear is in the staining or alcohol solutions. During clearing, alcohol is being replaced with xylene, which is also miscible in mounting medium. Xylene has a refractive index as that of glass and mounting medium and it prevents cellular distortion.
6. **Mounting of Slide.** The mounting media must be miscible with the clearing agent to prevent fading of the stains. Practice is essential to achieve well-mounted slides, free of air bubbles and artifacts. A minimum of mounting medium should be used. Too much mounting medium interferes with microscopic detail, making the cell film appear hazy or milky when examined under the high power objective. If the mounting medium and cover slip are applied too slowly, a common artifact appears as a brown refractile pigment like substance on the surface of the cell when xylene evaporates. If this artifact occurs, the slide must be soaked in xylene, absolute alcohol and 95% alcohol, rinsed in running tap water and restained in OG and EA. A possible means of preventing the 'brown artifact' is to coverslip slide behind a transparent chemical splash shield set at the front edge of the fume hood. The shield diverts air around the local workspace and reduces the rate of xylene evaporation. The usual size of the coverslip for a cervical smear is 22 X 30mm. If the smear spread is beyond the coverslip area, ideally use another small coverslip or put a drop of DPX and spread evenly with the same coverslip without affecting the focus.

Precautions

- Immediate fixation of smears is essential.
- Smears should never be allowed to dry before placing the coverslip.
- Hematoxylin is filtered everyday before use.
- All solutions and stains are filtered before use, to keep them free of sediment.
- Avoid contamination from one smear to another.
- Keep stains and solutions covered when not in use.
- All dishes are washed daily.
- Stains are discarded and replaced as the quality of the stain deteriorates.
- Avoid contamination during placing of the coverslip, with the dropper used to dispense the mounting medium.
- Place the coverslip on the microslide slowly without trapping air bubbles.

Maintenance of Stains and Solutions

- Solutions may be used for longer period of time, if the slide carrier is rested on several layers of tissue paper (paper toweling) for a few seconds before transferring to the solutions.
- Stains keep longer if they are stored in dark colored, stoppered bottles.
- Hematoxylin keeps relatively constant staining characteristics and do not require frequent discarding if small amounts of fresh stain are added to replace stain loss due to evaporation.
- Use of coating or spray fixatives may cause contamination making frequent changes necessary.
- OG and EA stains lose strength more rapidly than hematoxylin and should be replaced each week or as soon as the cells appear without crisp staining colors.
- Bluing solution and HCl should be replaced at least once daily.
- Water rinses should be changed after each use.
- Alcohol used for the process of dehydration prior to the cytoplasmic stains may be replaced weekly. The alcohol rinses following the cytoplasmic stains are usually changed on a rotating basis after each use. The alcohol rinse immediately following the stain is discarded, and the other two rinses are moved to the first and second position, and fresh unused alcohol is replaced in third position. Ideally this rotation must continue after each staining run. The absolute alcohols should be changed weekly and can be kept water free by adding silica gel pellets.
- Xylene should be changed as soon as it becomes tinted with any of the cytoplasmic stains. Xylene becomes slightly milky if water is present in it and if so the clearing process may be disturbed. Tiny drops of water may be seen microscopically on a plane above the cell on a slide. Addition of silica gel pellets to the absolute alcohol will minimize water contamination of xylene.
- Agitation of the slides by occasional dipping is necessary to remove excess dye.
- Dipping should be done gently to avoid cell loss and the slide carrier should not hit the bottom of the staining dish.
- The quality of the stained slide is dependent on timing, solubility and percentage of dye concentration.

RAPID PAPANICOLAOU STAINING

The purpose is to save staining time and money by combining OG and EA and reducing the number of rinses. This procedure needs to be done only for emergency situations and not for routine use.

Contamination Control

All stains, hematoxylin, OG-6 and EA-36 should be filtered at least once daily. The alcohols used for rehydration, dehydration, and xylene must be filtered or replaced daily. Gynecological and non-gynecological materials may be stained separately. Specimens prone for shedding cells and those suspected to have large number of cancer cell should be stained at the end of the day using separate rack. Even with all these precautions, gross contamination may occur, and if this happens with malignant cells all solutions and stains must be immediately filtered or discarded.

HEMATOXYLIN AND EOSIN (H&E) STAINING METHOD

Some laboratories use routine H&E stain for non-gynecological smears. The benefits of using Papanicolaou stain are clear definition of nuclear details and differential counterstaining giving cytoplasmic transparency. H&E stain does not satisfy these criteria and hence unacceptable for cervical smears.

MAY–GRUNWALD–GIEMSA (MGG) STAINING METHOD

Many laboratories use MGG (Romanowski type stain) staining method for cytological diagnosis of non-gynecological specimens in addition to Pap and H&E stains. Combination of all these stains increases the efficiency of microscopic interpretations. MGG stain is performed on air-dried aspirates or fluids. Stock solutions of May–Grunwald reagent and Giemsa stain are available commercially.

Staining Procedure

1. May–Grunwald solution – 5 mt.
2. Running water – 1 mt.
3. Giemsa solution – 15 mt.
4. Running water – 1–2 mt.
5. Air-dry (no mounting necessary)

Labeling of Slides

After the slides have been cleaned, they are ready for labeling. Place a small square label on the edge of the slide on the same side as the cover slip. Use waterproof ink and record the institution, details, accession, year, nature of specimen, etc. on it.

Filing the Slides

The slides must be protected from breakage, light, moisture and dust. After microscopic interpretation, the slides must be filed in slide filing cabinets in serial order, in numbered slots. They are kept for a minimum of 5 years and are retrieved when necessary.

NOTES ON IMMUNOHISTOCHEMISTRY

Immunohistochemistry is the localization of antigens in tissue sections by the use of labeled antibody as specific reagents through antigen–antibody interactions that are visualized by a marker such as fluorescent dye, enzyme, radioactive element or colloidal gold.

Albert H Coons and his colleagues (Coons et al. 1941, 1955; Coons and Kaplan 1950) were the first to label antibodies with a fluorescent dye, and use it to identify antigens in tissue sections. With the expansion and development of immunohistochemistry techniques, enzyme labels have been introduced such as peroxidase (Nakane and Pierce 1966; Avrameas and Uriel 1966) and alkaline phosphatase (Mason and Sammons 1978). Colloidal gold (Faulk and Taylor 1971) label has also been discovered and used to identify immunohistochemical reactions at both light and electron microscopy level. Other labels include radioactive elements, and immunoreaction can be visualized by autoradiography.

Since immunohistochemistry involves specific antigen–antibody reaction, it has apparent advantage over traditionally used special enzyme staining techniques that identify only a limited number of proteins, enzymes and tissue structures. Therefore, immunohistochemistry has become a crucial technique and widely used in many medical research laboratories as well as clinical diagnostics.

There are numerous immunohistochemistry methods that may be used to localize antigens. The selection of a suitable method should be based on parameters such as the type of specimen under investigation and the degree of sensitivity and specificity required.

FIXATION

Tissue preparation is the cornerstone of immunohistochemistry. To ensure the preservation of tissue architecture and cell morphology, prompt and adequate fixation is essential. However, inappropriate or prolonged fixation may significantly diminish the antibody binding capability.

There is no one universal fixative that is ideal for the demonstration of all antigens. However, in general, many antigens can be successfully demonstrated in formalin-fixed paraffin-embedded tissue sections. The discovery and development of antigen retrieval techniques further enhanced the use of formalin as routine fixative for immunohistochemistry in many research laboratories.

For best results, vertebrate tissues (especially neuronal tissues) usually require fixation by transcardial perfusion for optimal tissue preservation. The most common fixatives used for immunohistochemistry are:

1. 4% paraformaldehyde in 0.1M phosphate buffer
2. 2% paraformaldehyde with 0.2% picric acid in 0.1M phosphate buffer
3. PLP fixative: 4% paraformaldehyde, 0.2% periodate and 1.2% lysine in 0.1M phosphate buffer

4. 4% paraformaldehyde with 0.05% glutaraldehyde (TEM immunohistochemistry).

Some antigens will not survive even moderate amounts of aldehyde fixation. Under this condition, tissues should be rapidly fresh frozen in liquid nitrogen (snap frozen) and cut with a cryostat. The sections should be kept frozen at –20° C or lower until fixation with cold acetone or alcohol. After fixation, the sections can be processed using standard immunohistochemical staining protocols.

SECTIONING

Since its introduction, paraffin wax has remained the most widely used embedding medium for diagnostic histopathology in routine histological laboratories. Accordingly, the largest proportion of material for immunohistochemistry is formalin-fixed, paraffin-embedded. Paraffin sections produce satisfactory results for the demonstration of majority of tissue antigens with the use of antigen retrieval techniques.

Certain cell antigens do not survive routine fixation and paraffin embedding. So, the use of frozen sections still remains essential for the demonstration of many antigens. However, the disadvantages of frozen sections include poor morphology, poor resolution at higher magnifications, special storage needed, limited retrospective studies, and cutting difficulty over paraffin sections.

Vibratome sections have some advantages when doing immunohistochemistry since the tissue is not processed through organic solvents or high heat, which can destroy the antigenicity. In addition, the morphology of tissue sections are not disrupted since no freezing and thawing needed. Vibratome sections are often used for floating immunostaining, especially for pre-embedding EM immunohistochemistry. The disadvantage of vibratome sections is that the sectioning process is slow and difficult with soft and poorly fixed tissues. In addition, the chatter marks or vibratome lines often appears in the sections.

WHOLE MOUNT PREPARATION

Small blocks of tissue (less than 5 mm thick) can be processed as whole mounts. The advantage of whole mount preparations is that the results provide three- dimensional information about the location of antigens without the need for reconstruction from sections. However, the major limitation of using whole mounts is antibody penetration may not be complete in the tissue, resulting in uneven staining or false negative staining. So Triton X-100 or saponin treatment is used routinely for whole mount immunohistochemistry to enhance penetration of the antibody.

The demonstration of many antigens can be significantly improved by pretreatment with the antigen retrieval reagent that breaks the protein cross-links formed by formalin fixation and thereby uncover hidden antigenic sites. The techniques involved the application of heat for varying lengths of time to formalin-fixed, paraffin-embedded tissue sections in an aqueous solution (commonly referred to as the retrieval solution). This is called 'heat induced epitope retrieval (HIER)'. Another method uses enzyme digestion and is called proteolysis induced epitope retrieval (PIER).

Microwave oven, pressure cooker and steamer are the most commonly used heating devices. Other devices also include the use of autoclave and water bath. The heating length of 20 minutes appears to be the most satisfactory and the cooling usually takes about 20 minutes. **Citrate buffer of pH 6.0** is the most popularly used retrieval solution and is suitable for most of antibody applications. The **TRIS-EDTA of pH 9.0** and **EDTA of pH 8.0** are second most used retrieval solutions. **Proteinase K** is effective enzyme digestion reagent for membrane antigens such as integrins, CD31, vWF, etc.

PIER methods (such as **proteinase k**, **trypsin**, chymotrypsin, **pepsin**, **pronase** and various other proteases) have also been reported for restoring immunoreactivity to tissue antigens with different degrees of success. However, the use of enzyme digestion method may destroy some epitopes and tissue morphology. Therefore the optimal enzyme concentration and incubation time need to be tested.

Combination of heat mediated and proteolytic enzyme method is an alternative approach to unmask antigens if other methods did not work. It is especially useful when performing double or triple labeling of two or more antigens simultaneously.

Improving antibody penetration is also important for immunohistochemical staining of frozen and vibratome sections. Triton X-100 is by far the most popular detergent for improving antibody penetration for immunohistochemistry. However, it is not appropriate for the use of membrane antigens since triton X-100 destroy membranes. Some researchers prefer the freeze and thaw method for the improvement of antibody penetration. Sodium borohydride (1% in phosphate buffer) treatment is also widely used to unmask antigens, particularly in glutaraldehyde fixed tissue to reduce the glutaraldehyde linkages.

BLOCKING

Background staining may be specific or non-specific. Inadequate or delayed fixation may give rise to false positive results due to the passive uptake of serum protein and diffusion of the antigen. Such false positives are common in the centre of large tissue blocks or throughout tissues in which fixation was delayed.

Antibodies, especially polyclonal antibodies are sometimes contaminated with other antibodies due to impure antigen used to immunize the host animal.

The main cause of non-specific background staining is non-immunological binding of the specific immune sera by hydrophobic and electrostatic forces to certain sites within tissue sections. This form of background staining is usually uniform and can be reduced by blocking those sites with normal serum.

Endogenous peroxidase activity is found in many tissues and can be detected by reacting fixed tissue sections with DAB substrate. The solution for eliminating endogenous peroxidase

activity is by the pretreatment of the tissue section with hydrogen peroxide prior to incubation with primary antibody.

Many tissues also contain endogenous alkaline phosphatase (AP) activity and should be blocked by the pretreatment of the tissue section with levamisole if using AP as a label.

Some tissues such as liver and kidney have endogenous biotin. To avoid unwanted avidin binding to endogenous biotin if using biotin–avidin detection system, a step is necessary for these tissues by the pretreatment of unconjugated avidin which is then saturated with biotin.

Autofluorescence or natural fluorescence exists in some tissues and can cause background problems when fluorescent dyes are used in the experiments. The simplest test is to view the tissue sections with a fluorescence microscope before any antibody incubation. If autofluorescence is detected in the tissue sections, the best solution is to avoid use of fluorescent method but select enzyme or other labeling methods.

CONTROLS

Special controls must be run in order to test the protocol and for the specificity of the antibody being used.

Positive control is to test a protocol or procedure and make sure it works. It will be ideal to use the tissue of known positive as a control. If the positive control tissue showed negative staining, the protocol or procedure needs to be checked until a good positive staining is obtained.

Negative control is to test for the specificity of an antibody involved. First, no staining must be shown when omitting primary antibody or replacing a specific primary antibody with normal serum (must be from the same species as primary antibody). This control is easy to achieve and can be used routinely in immunohistochemical staining.

Second, the staining must be inhibited by adsorption of a primary antibody with the purified antigen prior to its use, but not by adsorption with other related or unrelated antigens. This type of negative control is ideal and necessary in the characterization and evaluation of new antibodies but it is sometimes difficult to obtain the purified antigen, therefore it is rarely used routinely in immunohistochemical staining.

DIRECT METHOD

Direct method is one-step staining method, and involves a labeled antibody (i.e. labeled conjugated antiserum) reacting directly with the antigen in tissue sections. This technique utilizes only one antibody and the procedure is short and quick. However, it is insensitive due to little signal amplification and rarely used since the introduction of indirect method.

INDIRECT METHOD

Indirect method involves an unlabeled primary antibody (first layer) which reacts with tissue antigen, and a labeled secondary antibody (second layer) that reacts with primary antibody (Note: The secondary antibody must be against the IgG of the animal species in which the primary antibody has been raised, i.e. raised in a different species of animal e.g. rabbit, sheep). This method is more sensitive due to signal amplification through several secondary antibody reactions with different antigenic sites on the primary antibody. In addition, it is also economic since one labeled second layer antibody can be used with many first layer antibodies (raised from the same animal species) to different antigens.

The second layer antibody can be labeled with a fluorescent dye such as FITC, rhodamine or Texas red, and this is called indirect immunofluorescence method. The second layer antibody may be labeled with an enzyme such as peroxidase, alkaline phosphatase or glucose oxidase, and this is called indirect immunoenzyme method.

PAP METHOD

Peroxidase Anti-peroxidase Method (PAP) method is a further development of the indirect technique and it involves a third layer which is a rabbit antibody to peroxidase, coupled with peroxidase to make a very stable peroxidase anti-peroxidase complex. The complex, composed of rabbit gamma-globulin and peroxidase, acts as a third layer antigen and becomes bound to the unconjugated goat anti-rabbit gamma-globulin of the second layer. The sensitivity is about 100 to 1000 times higher since the peroxidase molecule is not chemically conjugated to the anti IgG but immunologically bound, and loses none of its enzyme activity. It also allows for much higher dilution of the primary antibody, thus eliminating many of the unwanted antibodies and reducing non-specific background staining.

ABC METHOD

Avidin-Biotin Complex (ABC) method is standard immunohistochemistry (IHC) method and one of widely used techniques for immunohistochemical staining. Avidin, a large glycoprotein, can be labeled with peroxidase or fluorescein and has a very high affinity for biotin. Biotin, a low molecular weight vitamin, can be conjugated to a variety of biological molecules such as antibodies.

The technique involves three layers. The first layer is unlabeled primary antibody. The second layer is biotinylated secondary antibody. The third layer is a complex of avidin-biotin peroxidase. The peroxidase is then developed by the diaminobenzidine tetrahydrochloride (DAB) or other substrate to produce differently colored end products.

LSAB METHOD

Streptavidin, derived from *Streptococcus avidinii*, is a recent innovation for substitution of avidin. The streptavidin molecule is uncharged relative to animal tissue, unlike avidin which has an isoelectric point of 10, and therefore electrostatic binding to tissue is eliminated. In addition, streptavidin does not contain carbohydrate groups which might bind to tissue lectins, resulting in some background staining.

Labeled streptavidin biotin (LSAB) is technically similar to standard ABC method. The first layer is unlabeled primary

antibody. The second layer is biotinylated secondary antibody. The third layer is Enzyme-Streptavidin conjugates horseradish peroxidase; AD–Alkaline phosphatase (HRP*-Streptavidin or AP*-Streptavidin) to replace the complex of avidin-biotin peroxidase. The enzyme is then visualized by application of the substrate chromogen solutions to produce different colored end products. The third layer can also be fluorescent dye-Streptavidin such as FITC-Streptavidin if fluorescence labeling is preferred.

A recent report suggests that LSAB method is about 5 to 10 times more sensitive than standard ABC method.

POLYMERIC METHODS

These are based on dextran polymer technology. This unique chemistry permits binding of a large number of enzyme molecules (horseradish peroxidase or alkaline phosphatase) to a secondary antibody via the dextran backbone. The benefits are many, including increased sensitivity, minimized non-specific background staining, and a reduction in the total number of assay steps as compared to conventional techniques. The simple protocol is: (i) application of primary antibody, (ii) application of enzyme labeled polymer, and (iii) application of the substrate chromogen.

Polymerized Reporter Enzyme Staining System is based on a new method of polymerizing enzymes and attaching these polymers to antibodies. The novel approach employed to form enzyme 'micropolymers' avoids the intrinsic shortcomings of using large dextrans or other macromolecules as backbones. Attaching a unique micropolymer with a high density of very active enzyme to a secondary antibody generates a reagent that overcomes steric interference and provides enhanced accessibility to its target. The result is outstanding sensitivity, signal intensity, low background staining, and reduced non-specific binding. The simple protocol is: (i) application of primary antibody, (ii) application of enzyme labeled polymer, and (iii) application of the substrate chromogen.

Tyramide Signal Amplification (TSA). It is ideal for the following applications: (i) detecting small quantities of antigen, (ii) enhancing performance of low affinity mouse and rabbit antibodies, and (iii) enabling compatibility of certain 'tough' mouse and rabbit antibodies with paraffin embedded tissue sections. The simple protocol is as follows:

1. Application of primary antibody
2. Application of biotinylated linking antibody
3. Application of the tyramide amplification reagent
4. Application of streptavidin-HRP
5. Application of the substrate chromogen.

Biotin-free TSA System is a highly sensitive immunohistochemical (IHC) staining procedure incorporating a signal amplification method based on the peroxidase-catalyzed deposition of a fluorescein-labeled phenolic compound, followed by a secondary reaction with a peroxidase-conjugated anti-fluorescein. In the procedure, a mouse primary antibody is first detected with a peroxidase-conjugated secondary antibody. The next step utilizes the bound peroxidase to catalyze oxidation of a fluorescein-conjugated phenol (fluorescyl-tyramide) which then precipitates onto the specimen. The procedure is continued with detection of the bound fluorescein by a peroxidase-conjugated anti-fluorescein. Staining is completed using diaminobenzidine/hydrogen peroxide as chromogen/substrate, and can be observed with a light microscope. In comparison to standard immunohistochemical methods, such as labeled streptavidin biotin (LSAB) or avidin-biotin complexes (ABC), tyramide amplification methods have been reported to be many fold more sensitive. This reagent system utilizes fluorescyl-tyramide, rather than biotinyl-tyramide, and does not contain avidin/biotin reagents, thus eliminating potential background staining due to reactivity with endogenous biotin.

Principles of Procedure. The specimens are first incubated with peroxidase block for 5 minutes to quench endogenous peroxidase activity. The specimens are then incubated for 5 minutes with a protein block to suppress nonspecific binding of subsequent reagents, followed by a 15-minute incubation with an appropriately characterized and diluted mouse primary antibody or negative control reagent (user provided). This is followed by sequential 15-minute incubations with anti-mouse immunoglobulins-HRP, fluorescyl-tyramide hydrogen peroxide (amplification reagent) and anti-fluorescein-HRP. Staining is completed by a 5-minute incubation with 3,3' diaminobenzidine tetrahydrochloride (DAB)/hydrogen peroxide, which results in a brown precipitate at the antigen site.

MULTIPLE LABELING

It is often useful to be able to stain for two or more antigens in one common tissue section. This can be achieved by immunofluorescence method using different fluorescent dyes. Multiple staining can also be done with peroxidase conjugated antibodies developed with different chromogen substrates to produce the end products of different colors. There are three basic approaches in planning multiple staining: parallel, sequential and adjacent. In addition, the antibody dilution and conditions are also important factors to be considered. Finally, appropriate color combination is also crucial since improper color combination may produce poor result and fail to demonstrate multiple antigens in the same section. For best result, the careful design and test of multiple staining protocols are necessary.

Immuno-Electron Microscopy

Electron microscopic (EM) immunohistochemical techniques can be divided into two groups: (i) those where the immunostaining takes place prior to resin embedding are referred to as pre-embedding, and (ii) those methods where the immunolabeling is undertaken after resin embedding are known as post-embedding.

The choice of whether to apply pre- or post-embedding method to the detection of an antigen in any particular location will depend to a large extent upon the distribution and availability of the antigen and the characteristics of the primary antibody. Before starting immuno-EM labeling, a test

for the characteristics and dilution of the primary antibody should be performed at light microcopy level.

Several recently developed methods rely on labeling with colloidal gold particles. These methods were originally introduced for electron microscopy (Faulk and Taylor 1971) as the gold particles are easily visible under the electron microscope, but they are also useful for light microscopy.

Since gold particles can be made in different sizes from 5 to 30 nm, it is possible to carry out multiple staining at the electron microscopic level, most easily by direct labeling of several first layer antibodies with different sized particles. The indirect techniques can also be used in double or triple labeling by parallel approach if the primary antibodies are from different species and by sequential approach if the primary antibodies are from same species.

STANDARD IHC METHOD

1. Cut paraffin sections and mount on adhesive slides.
2. Deparaffinize slides in xylene and graded alcohols to water.
3. Quench endogenous peroxidase activity. Rinse in buffer.
4. Incubate with 'blocking serum'. Rinse in buffer.
5. Perform epitope retrieval step (antigen retrieval or protease) if needed. Rinse in buffer.
6. Apply primary antibody, incubate, and rinse in buffer.
7. Apply secondary (link) antibody, incubate, and rinse in buffer.
8. Apply detection complex, incubate, and rinse in buffer.
9. Develop reaction product with chromogen, counterstain, dehydrate, coverslip, and view.

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