Histology Manual

Karmen Michael Owen

Embedding SOP

IRF Instrumentation Resource Facility - University of South Carolina School of Medicine
Standard Operating Procedure (SOP) for Embedding Tissues
Thermo Scientific TN1550 Embedding Center
Location: IRF Lab Room B60

REQUIREMENTS

All users are required to undergo proper embedding training. All users must read and sign the microtome SOP before operating the instrument independently. A list of trained individuals is to be kept by the histology manager. To schedule time to embed tissues, contact the histology manager.

If there is any issue with the embedding center, the histology manager should be notified:

This instrument is maintained by Karmen Owen
Email: Karmen.Owen@uscmed.sc.edu
Cell Phone: (803) 237-9583

TEMPERATURE

Molten wax is kept at 60°C therefore the station will be heated up and will be hot. Avoid touching surfaces without gloves on to prevent burns. An apron or lab coat may be used to protect your clothing from paraffin splashes.

EMBEDDING PROCEDURE

1. To turn on the embedding station, the switch on the back should be flipped on. The arrow button on the display should then be pressed to turn on “manual” mode.
2. The cooling station switch should be turned on. It takes about 15 minutes to cool down.
3. After tissues are fully processed, they should be transferred to the embedding station chamber.
4. A mold should be picked that is the appropriate size for the specimen. The molds are in the other chamber or extra molds are in the drawer underneath the embedding station.
5. Choose a cassette from the chamber to start to embed. Remove the lid from the cassette and throw it away.
6. Push back the lever to dispense paraffin into the mold.
7. If the machine is running low on paraffin, paraffin pellets can be placed in the back reservoir of the embedding station.
8. Place the specimen in the mold with heated forceps.

9. Orient the specimen properly. Use the cold plate to slightly solidify the paraffin to the bottom of the mold which can then be used to ‘hold’ the specimen in place. The mini cold plate on this instrument only works a little bit. The cooling station can also be used.
10. Place the now empty cassette on top of the embedding mold and be sure it is seated properly.
11. Fill the top of the cassette with more paraffin.
12. Carefully place the embedded specimen on to the cold plate in an orderly manner.
13. Blocks can be removed from the molds when they have completely solidified and are cold to the touch.
14. Blocks should come out of the mold easily, do not try to force a block out of the mold.
15. Repeat procedure with all specimens.

SHUT DOWN

1. Remove the collection trays under both chambers of the embedding center and empty the used paraffin into the trash can.
2. Using a spatula, scrape the extra paraffin from around the hot plates and discard in the trash can.
3. Use gauze to wipe down the area around the hot plates and rub the extra paraffin away from the outer surfaces of the embedding center.
4. After all of the blocks have been removed from the molds, replace the metal molds back into the chamber for reuse and close the chambers.
5. Flip the switch on the back of the embedding station to the ‘off’ position.
6. Switch the cold plate off.

SAFETY

In case of injury, seek first aid. A first aid kit is located at the front of the lab benches. An IRF staff member should be notified of the injury and an incident report should be filled out.

Microtome SOP

IRF Instrumentation Resource Facility - University of South Carolina School of Medicine
Standard Operating Procedure (SOP) for Microtome Sectioning
Reichert-Jung 2030 Rotary Microtome and Leica RM 2255 Automatic Microtome
Location: IRF Lab Room B60

REQUIREMENTS

All users are required to undergo proper microtome training. All users must read and sign the microtome SOP before operating the instrument independently. A list of trained individuals is to be kept by the histology manager. All users are required to log their usage time in the log book. Reserved time on the microtome can be booked through an iLabs account.

If there is any issue with histology, the histology manager should be notified:

This instrument is maintained by Karmen Owen
Email: Karmen.Owen@uscmed.sc.edu
Cell Phone: (803) 237-9583

PROCEDURE - SET UP

1. Tissue specimens should be properly embedded in paraffin wax. Blocks should then be placed on an ice block to keep them properly cooled.
2. Select the desired trimming thickness. To trim the block, the settings should be at around 10um and for sectioning the settings should be around 5um.
3. During cutting, the block holder moves up and down as the handwheel is rotated. After the up stroke, the block holder moves forward in increments of the set trimming thickness setting.
4. Make sure the handwheel is rotated until the specimen clamp is in the uppermost position.
5. Insert the block into the block holder by pushing the lever towards you. Release the handle to secure the block. A block can be positioned horizontally or vertically.
6. Insert a new blade into the blade holder CAREFULLY and tighten the blade holder. Angle the blade and specimen so that the block face is parallel to the knife edge.
7. Make sure all bolts are tightened before unlocking the handwheel.
8. Use the handwheel to advance the knife forwards to move the specimen close to the knife.

Different sections of the blade can be used at different times. If there are blade marks starting to show while sectioning, move the blade down to a newer section of blade. Start at one side of the blade and then move the blade down when it needs to be changed.

SECTIONING

1. Start to turn the wheel slowly to see how close the blade is to the block. Advance or retract the specimen block based on what is needed.
2. Blocks should be faced to expose the entire tissue by trimming it at 5-10µm. Continue trimming by rotating the microtome wheel until the specimen is fully exposed to the point desired for sections.
3. After facing the block, put the block back on ice to keep them cold before sectioning. When ready to start sectioning, the section thickness should be set to 5µm.
4. After trimming move the blade to a different portion of blade to start sectioning.
5. Keep rotating the hand wheel clockwise to start making sections. A ribbon should start forming.
6. Gently, pick up the ribbon and lay it on top of the water bath.
7. Multiple sections can fit on to a slide. Orientation and location of the tissue should be considered before placing the tissue.
8. To change blocks, move the specimen clamp to the upper end position and activate the handwheel lock. Remove the blade before changing blocks.
9. Always keep the knife guard up and the hand wheel locked when not actively using the microtome.

WATER BATH

1. Fill a water bath with distilled water and let it heat to 10° less than the melting point of the paraffin being used.
2. Gently, pick up the ribbon and let it float on top of a heated water bath. The sections should flatten out.
3. A long ribbon can be separated in the bath into individual sections.
4. Orient a glass slide next to a section and slip the slide under the water at a 45° angle. Move the slide underneath the wanted section and touch it to the ribbon.
5. Lift the slide up at an angle.
6. After the desired sections are on slides, let the slides air-dry.
7. Make sure to turn off the water bath after use.

CLEANING

1. When you are done sectioning, lock the handwheel with the specimen holder in the top position.
2. Remove the blade from the knife holder and discard it in the sharps container.
3. Remove the block from the block holder.
4. Brush loose paraffin into the waste paraffin tray with gauze. Empty the paraffin tray into the trash by pulling it out until the magnets release the tray. Return the empty paraffin tray.
5. Unwanted paraffin trimmings can be disposed of in a regular trash bin.
6. Paraffin can be wiped from the area using gauze. Wipe down the microtome and water bath with 70% ethanol on a Kimwipe.
7. Empty ice bath in the sink and replace with water and return it back to the freezer.
8. Empty the water bath in the sink and make sure it is turned off
9. Remember to record your time in the log book.

STAINING

1. Air dry the slides at room temperature.
2. Place in an oven at 70° for at least 20 minutes to melt the paraffin. This is an essential step, as otherwise the sections will fall off the slide.
3. Stain the slides using whichever stain protocol is wanted.
4. Coverslip slides in the coverslipper machine using mounting media and glass coverslips.

BLADES

Use with caution!! Microtome blades are very sharp and can cause serious injuries. Never touch a blade with your fingers. Always lock the handwheel when manipulating the blade. Do not clean the blade along its length. Wipe the blade from the back edge to the cutting edge with a brush when cleaning. Put blade guard up when not sectioning. Never leave the microtome unattended with an exposed blade in position. If you need to step away, remove the blade or use the blade guard.
After Use: Remove the blade when you have finished using the microtome. Never leave a used blade in the blade holder in the microtome. All used blades should be properly disposed of in the appropriate sharps container located on the lab bench. Do not place blades in the trashcan or laying on the benchtop.

WASTE MANAGEMENT

All trimmings and sections of tissue that accumulate on the microtome should be brushed into the waste container below the microtome. This waste container with all of the trimmings should be emptied into a trashcan. All blades should be disposed of properly in the designated sharps container on the lab bench.

PREVENTATIVE MAINTENANCE AND CLEANING

Before cleaning the instrument, make sure to lock the rotary hand wheel. Make sure all blades are removed and disposed of. All surfaces of the microtome can be disinfected using 70% ethanol. Gauze can help get the paraffin off the microtome.

SAFETY

In case of injury, seek first aid. A first aid kit is located at the front of the lab benches. An IRF staff member should be notified of the injury and an incident report should be filled out.

CRYOSTAT SOP

IRF Instrumentation Resource Facility - University of South Carolina School of Medicine
Standard Operating Procedure (SOP) for Frozen Sectioning
Thermo Scientific Cryostat Microm HM 525
Location: IRF Lab Room B60

REQUIREMENTS

All users are required to undergo proper cryostat training. All users must read and sign the cryostat SOP before operating the instrument independently. A list of trained individuals is to be kept by the histology manager. All users are required to log their usage time in the log book. Reserved time on the cryostat can be booked through an iLabs account.

If there is any issue with the cryostat, the histology manager should be notified:

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Cell Phone: (803) 237-9583

PROCEDURE - SET UP

9. Samples should be brought down to the core on ice or dry ice.
10. Pick the desired mold for your sample. Place the sample in the mold face down and cover it with OCT media until the entire well is covered. Avoid air bubbles. Put it in the cryostat and let it freeze.
11. Once it is completely frozen and is white in color, it can be popped out of the mold. Apply a small amount of OCT on the specimen chuck and mount the specimen onto it. Allow OCT media to harden. Let it briefly freeze before mounting it.
12. Turn the cryostat light on. Lock the handwheel in the upper position before working with hands inside the cryostat.
13. Insert the chuck into the specimen holder and tighten the screw. The specimen can be manipulated by untightening the clamp on the right side of the specimen holder.
14. Insert new blade into the blade holder CAREFULLY and tighten the blade holder. Angle the blade and specimen so that the block face is parallel to the knife edge.

> The knife angle on this cryostat sometimes does not stay in the correct position. Make sure to set the angle tightly at 5-6 µm and avoid hitting the clamp during

15. Select the desired trimming thickness. The most common thickness for frozen sectioning is
16. 10um. During cutting, the block holder moves up and down as the handwheel is rotated. After the up stroke, the block holder moves forward in increments of the set trimming thickness setting.
17. Make sure all bolts are tightened before unlocking the handwheel.
18. Press the motorized coarse feed button to move the specimen close to the knife.

SECTIONING

10. Keep your samples in the cryostat until they are ready to be sectioned. Do not allow the tissue to thaw at any time. It is best to only work with one sample at a time.
11. During trimming, keep the antiroll device up. Advance the knife by turning the handwheel and start trimming the block face. Continue trimming until the specimen is exposed to the point desired for sections. Discard the first 2-3 sections to ensure that desired thickness is achieved.
12. Keep the knife and specimen free of debris by brushing upwards with a brush.
13. When ready to section, lower the antiroll device glass onto the stage in the correct position. This will catch the sections as they are cut.
14. Continue to advance the knife to start taking sections. When a desired section is cut, lift the antiroll plate and touch a glass slide face down onto the section. The section should jump and stick onto the slide.
15. Multiple sections can fit on to a slide. Orientation and location of the tissue should be considered before placing the tissue. If a section needs to be repositioned in any way, a brush can be used to manipulate the section.
16. The slides can be stored in the cryostat while sectioning. Let the slides dry at room temperature before staining.

SHUT DOWN

1. When you are done sectioning, lock the handwheel with the specimen holder in the top position. Press the rewind button to move the specimen holder all the way to the back, to the rear position.
2. Remove the blade from the knife holder and discard it in the sharps container.
3. Remove the chuck from the specimen holder and also remove the specimen from the chuck. The specimen can also be removed with running warm water. Clean the chuck with water at the sink or 70% ethanol and wipe the chuck with a Kimwipe.
4. The cryostat chamber and accessories can be cleaned using 70% ethanol on a Kimwipe.
5. Make sure the window is down and the chamber light is off. Leave the main power on. Do not shut down the main power of the instrument.

STORAGE

Slides can be stored in -20°C if being used in the next few weeks and should be stored in -80°C if they are going to be stored for months or years at a time. The slides should only be thawed when they are ready to be used.

BLADES

Use with caution!! Microtome blades are very sharp and can cause serious injuries. Never touch a blade with your fingers. Always lock the handwheel when manipulating the blade. Do not clean the blade along its length. Wipe the blade from the back edge to the cutting edge with a brush when cleaning. Put blade guard up when not sectioning. Never leave the microtome unattended with an exposed blade in position. If you need to step away, remove the blade or use the blade guard.

After Use: Remove the blade when you have finished using the cryostat. Never leave a used blade in the blade holder in the cryostat. All used blades should be properly disposed of in the appropriate sharps container located on the lab bench. Do not place blades in the trashcan or laying on the benchtop.

TEMPERATURE

- Temperature range of the cryostat is -20°C to -35°C. Do not go above -20°C.
- Keep temperature of the cryostat on -23°C. If you change the temperature of the cryostat, put it back on -23°C when you are done using it.
- The cryostat operates at extremely low temperatures and is capable of causing frostbite. Be sure to wear gloves when working in the chamber.
- If sections are accumulating on the knife edge, the temperature may be too warm. Keep the glass door shut as much as possible. Freeze spray may also be used.
- If sections are curling, the temperature may be too cold. Place your finger on the specimen to warm it up.

WASTE MANAGEMENT

Do not leave specimens in the cryostat. The waste container below the blade holder should be emptied into a trashcan. All trimmings and sections of tissue that accumulate in the cryostat should be swept into the holes in the back of the cryostat. Any leftover specimens should be taken back to your own lab for disposal. All blades should be disposed of properly in the designated sharps container on the lab bench.

PREVENTATIVE MAINTENANCE AND CLEANING

Before cleaning the instrument, make sure to lock the rotary hand wheel. Make sure all blades are removed and disposed of. All surfaces of the cryostat can be disinfected using 70% ethanol. To defrost the cryostat, the main power should be turned off. All parts of the cryostat should be removed and cleaned separately. Everything should be thoroughly dried before being reinserted back into the chamber to prevent icing. The chamber should also be completely dried out before switching the cryostat back on.

SAFETY

In case of injury, seek first aid. A first aid kit is located at the front of the lab benches. An IRF staff member should be notified of the injury and an incident report should be filled out.

Instrumentation Used

Autostainer: Leica Autostainer XL

Leica ASP 300 Tissue Processor

Leica CV 5000 Automatic Coverslipper

Thermo Scientific Microm HM 525 Cryostat

Reichert-Jung 2030 Rotary Microtome

Thermo Scientific TN1550 Embedding Center

Nikon Eclipse E600 Light Microscope

Lab Vision PT Module for Antigen Retrieval

Leica RM 2255 Automatic Microtome (Karmen's)

Tissue Flotation Bath

Laboratory Oven

Histology

- Study of microscopic structure of cells, tissues, and organs
- Thin slice of tissue under a microscope

Steps of Histology

1. Fixation
2. Dehydration
3. Clearing
4. Paraffin Infiltration
5. Embedding
6. Sectioning
7. Drying
8. Staining

Processing

- Series of alcohols, xylene, and paraffin
- Overnight protocol
- (6 hours, 45 minutes)

Dehydration**:** the removal of water by increasing concentrations of ethanols

- Water molecules are removed from tissue

Clearing**:** ethanol is replaced by xylene

- Paraffin and ethanol are immiscible!

Paraffin Infiltration: tissue is infiltrated with a supporting medium

- Xylene is replaced with molten wax under vacuum

Staining

- H&E stain is the most common

Regressive staining – tissue is overstained with hematoxylin and differentiator is used to remove excess hematoxylin (Harris Hematoxylin, Weigert's Hematoxylin)

Progressive staining – tissue is stained with hematoxylin up to a point and excess hematoxylin is not removed (Mayer's Hematoxylin, Gill's Hematoxylin)

PAS Stain – Glycogen

Masson's Trichrome - Collagen

FIXATION

- Preserves tissues by stopping autolytic changes but allows tissues to remain unchanged by subsequent treatment.
- Functions to kill the tissue by denaturing proteins so that the postmortem activities of decay, or putrefaction (bacterial attack), and autolysis (enzyme attack) are prevented.
- Helps maintain the proper relationship between cells and extracellular substances.
- Tissues are hardened slightly.
- Resistant to further changes.
- Delayed fixation causes autolysis – fixation should be performed immediately after tissue has been removed.

FACTORS AFFECTING FIXATION

Temperature – Increase in temperature increases the rate of fixation but also increases rate of autolysis. Fixation should normally be done at room temperature. After 24 hours, can be stored in 4°C.

Size – Effects reagent penetration. Maximum size should be no more than 10mm for adequate fixation.

Volume Ratio – Fixative volume should be at least 20X greater than the tissue volume.

Fixative Used – Different fixatives penetrate at different rates. Most commonly used is 10% buffered neutral formalin or 4% paraformaldehyde (pH should be 7.2 to 7.4)

Time – Interval between interruption of the blood supply and placement of tissue in fixative should be minimal. Changes in tissue begin to occur within seconds so speed is essential.

Duration – 24 (small) to 48 hours (larger) (depends on size of tissue)

AFTER FIXATION

Rinse with distilled water and change to 70% ethanol. Can be stored long term in 4°C.

OVERNIGHT TISSUE PROCESSING PROTOCOL

Dehydration

- 70% Ethanol – 45 minutes
- 70% Ethanol – 30 minutes
- 95% Ethanol – 30 minutes
- 95% Ethanol – 30 minutes
- 95% Ethanol – 30 minutes
- 100% Ethanol – 20 minutes
- 100% Ethanol – 20 minutes
- 100% Ethanol – 20 minutes

Clearing

- Xylene – 20 minutes
- Xylene – 20 minutes
- Xylene – 20 minutes

Paraffin Infiltration

- Paraffin – 45 minutes
- Paraffin – 45 minutes
- Paraffin – 30 minutes

Routine H & E Protocol

1) *XYLENE - 5 minutes*
2) *XYLENE - 5 minutes*
3) *100% ETHANOL - 2 minute*
4) *100% ETHANOL - 2 minute*
5) *95% ETHANOL - 1 minute*
6) *95% ETHANOL - 1 minute*
7) *WASH 1 - WATER - 1 minute*
8) *HEMATOXYLIN - 6 minutes*
9) *WASH 2 - WATER – 5 minutes*
10) *WASH 3 - WATER - 2 minutes*
11) *ACID ALCOHOL - 2 seconds*
12) *WASH 4 - WATER - 5 minute*
13) *BLUING - 1 minute*
14) *WASH 5 - WATER - 5 minutes*
15) *95% ETHANOL - 2 minutes*
16) *EOSIN - 1 minute 30 seconds*
17) *95% ETHANOL - 30 seconds*
18) *95% ETHANOL - 30 seconds*
19) *100% ETHANOL - 1 minute*
20) *100% ETHANOL - 2 minute*
21) *100% ETHANOL - 1 minute*
22) *XYLENE - 5 minutes*
23) *XYLENE - 5 minutes*
24) *XYLENE - Exit*

Autostainer (H&E) Reagents

Bluing:

5 drops ammonia in water

Make fresh each day!

(Or use premade bluing reagent)

Hematoxylin:

Filter Daily

Eosin:

Stir daily

Acid Alcohol:

1% HCl in 70% ETOH

(1L) 10mL HCl and 990mL 70% ETOH

70% ETOH (1L):

700mL 100% ETOH

300mL H20

Switch reagents frequently

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(Frozen) Rapid H & E Protocol

25) *FORMALIN – 2 minutes*

26) *95% ETHANOL - 1 minute*

27) *WASH 2 – WATER – 20 seconds*

28) *WASH 3 – WATER – 20 seconds*

29) *HEMATOXYLIN – 1 minute*

30) *WASH 1 - 1 minute*

31) *ACID ALCOHOL – 2 seconds*

32) *WASH 4 - WATER – 10 seconds*

33) *BLUING – 10 seconds*

34) *WASH 5 - WATER – 30 seconds*

35) *95% ETHANOL – 5 seconds*

36) *EOSIN – 15 seconds*

37) *95% ETHANOL - 5 seconds*

38) *95% ETHANOL - 5 seconds*

39) *100% ETHANOL – 5 seconds*

40) *100% ETHANOL – 5 seconds*

41) *100% ETHANOL – 5 seconds*

42) *XYLENE – 5 seconds*

43) *XYLENE – 5 seconds*

44) *XYLENE - Exit*

Periodic Acid Schiff (PAS) Stain

Purpose: The Periodic Acid Schiff (PAS) stain is intended for use in demonstration of lymphocytes and mucopolysaccharides. It is also used to demonstrate glycogen, basement membranes, cellulose, and starch. PAS positive material will be stained magenta. Nuclei will be stained black or blue.

Positive Controls: Kidney, Intestine, Liver

Reagents: Periodic Acid Solution, Schiff's Solution, Mayer's Hematoxylin, Bluing Reagent

Principle: The periodic acid oxidizes the carbon to carbon bond forming aldehydes that react with the Schiff's solution, which forms the magenta color.

Use: The PAS stain is intended for use in histological demonstration of lymphocytes and mucopolysaccharides. The staining pattern of the lymphocytes are helpful in making therapeutic decisions in established cases of lymphocytic leukemia. The PAS stain can also be used for the demonstration of fungal organisms in tissue sections. Can be used to detect glycogen deposits in the liver when glycogen storage disease is suspected.

Periodic Acid Schiff (PAS) Stain

1. Deparaffinize and hydrate to water (program 3 on autostainer)
2. Immerse slide in Periodic Acid Solution for 5 minutes (7 minutes for kidney, skin and liver)

   Note: The Periodic Acid Solution and the Schiff's Solution are located in the refrigerator

3. Rinse in distilled water
4. Immerse slide in Schiff's Solution for 15 minutes (20 minutes for kidney, skin and liver)
5. Rinse slide in hot running tap water (beaker in oven)
6. Rinse slide in distilled water
7. Stain slide in Mayer's Hematoxylin for 1 minute
8. Rinse slide in running tap water for 2 minutes
9. Apply Bluing Reagent for 10 seconds
10. Rinse in distilled water
11. Dehydrate by using program 2 on the autostainer
12. Mount with mounting media and coverslip

Note: Save all reagents in original containers

Masson's Trichrome Stain

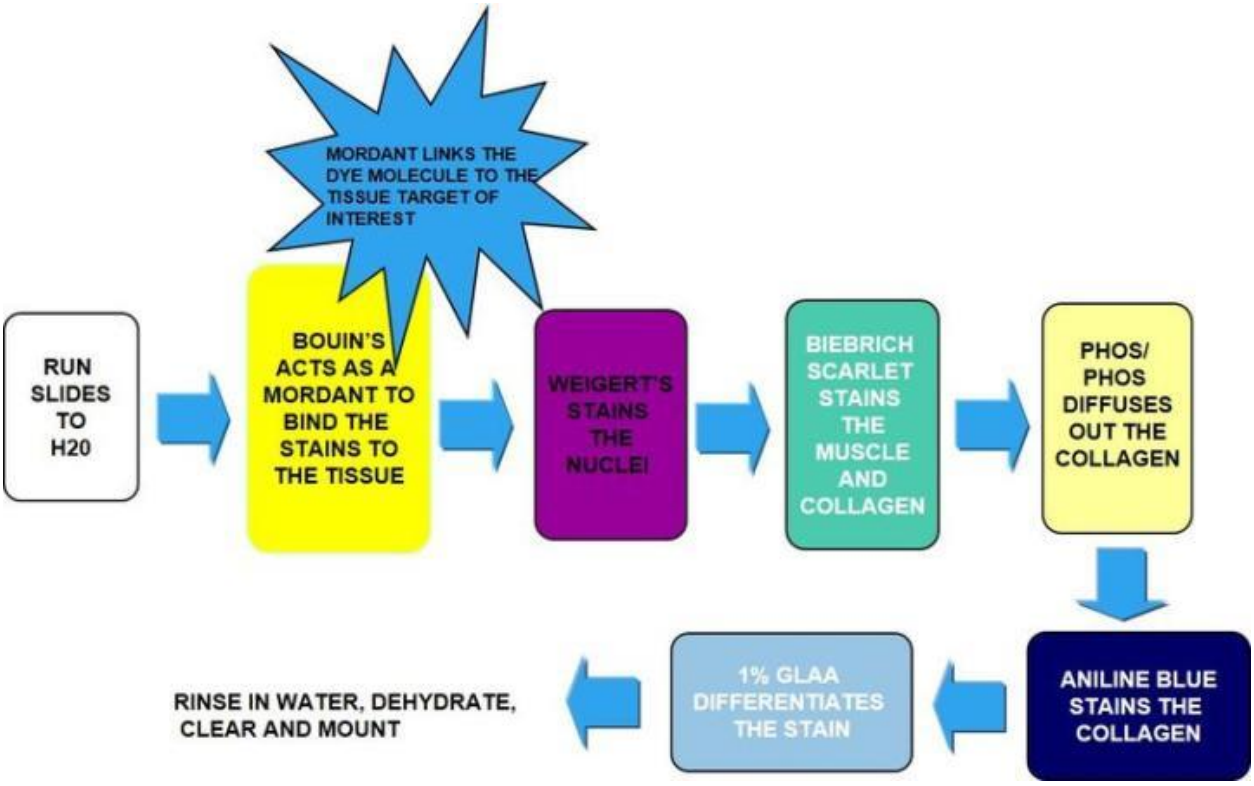
Purpose: Demonstration of collagen and smooth muscle. Collagen fibers will be stained blue. Nuclei will be stained black, and the muscle, cytoplasm, and keratin are stained red.

Positive Controls: Skin, Lung, Stomach, Intestine, Uterus, Appendix, Fallopian Tube

Reagents: Bouin's Solution, Weigert's Iron Hematoxylin Stock Solution A&B, Biebrich Scarlet-Acid Fuchsin Solution, Phosphomolybdic-Phosphotungstic Acid, Aniline Blue Solution, 1% Acetic Acid Solution

Principle: Post-fixation in Bouin's increases the intensity of the stain. The negatively charged nuclei are then stained with the positively charged hematoxylin. An acidic dye is applied to the section which binds all of the acidophilic elements including collagen and muscle. The phosphomolybdic-phosphotungstic acid then displaces the acid dye from the collagen. Collagen is more permeable than cytoplasm and the large phosphomolybdic-phosphotungstic molecule cannot displace the acid dye from the cytoplasm. It also acts as a mordant for the aniline blue to bind to collagen. Acetic acid helps clear the slide of any loosely bound dye.

Use: This method is used to differentiate between smooth muscle and of collagen fibers in tissue sections. This stain can assess the degree of fibrosis in the liver, which gives important information about stage and progression of a disease. Liver diseases such as hepatitis B and C viral infections, fatty liver disease, alcoholic liver disease and chronic biliary diseases show the formation of fibrous tissue including fibrous septa leading to the end-stage process called cirrhosis.



Masson's Trichrome Stain

1. Deparaffinize and hydrate to water (program 3 on autostainer)
2. Mordant in Bouin's fixative for 1 hour in the oven (70°)

> Note: Bouin's fixative has highly toxic fumes. Make sure fume hood is on and limit exposure. Close the lid on the container before putting it in the

3. Cool and wash in running water until yellow color disappears
4. Rinse in distilled water
5. Place in Weigert's Iron Hematoxylin Working Solution for 10 minutes

> Working Solution is made by adding equal parts of stock solution A and stock solution B. Mixture is good for 2 weeks. This step will be premade for you. When finished, pour mixture back into the original container.

6. Wash in running water for 10 minutes
7. Rinse in distilled water
8. Place in Biebrich Scarlet-Acid Fuchsin for 2 minutes. Save solution in original container when finished.
9. Rinse in distilled water
10. Place in phosphotungstic-Phosphomolybdic acid for 12 minutes.

> Note: Pour used acid in labeled waste container

11. Place in Aniline Blue solution for 5 minutes. Save solution in original container when finished
12. Rinse in distilled water
13. Place in acetic acid 1% aqueous for 4 minutes. Pour used acid in waste container.
14. Dehydrate by using program 2 on the autostainer
15. Mount with mounting media

Picrosirius Red Stain

Purpose: Stains fibrillar type I and type III collagen. Collagen will be stained red. Type I will be stained yellow, and type III will be stained green.

Positive Controls: Heart, Lung, Uterus, Muscle, Kidney

Reagents: Phosphomolybdic Acid, Picrosirius Red Stain, .1 N Hydrochloride Acid

Principle: Picric acid dye is a small and slightly hydrophobic molecule and Sirius Red is a red acid dye that is large and hydrophilic. This method stains collagen red, and cytoplasm and other protein rich material being stained different shades of yellow. Thick sections stain more yellow than thin sections.

Use: Picrosirius red method is used to stain collagen I and III. The stain will quantify the amount of collagen in a given area of myocardial tissue. Picrosirius Red Stain binds specifically to collagen fibrils of varying diameter that is used to distinguish collagen type I from collagen type III. Collagenous structures of the mandible stain brilliant red. Dentinal tubules, Sharpey's fibers and other structures not easily seen in sections stained with hematoxylin and eosin alone were seen clearly after this procedure. Under polarized light collagen fibers could be specifically identified and their orientation determined. Picrosirius Red Stain hematoxylin is recommended for examination of normal or pathologic dental specimens.

Picrosirius Red Stain

1. Deparaffinize and hydrate to water (program 3 on autostainer)
2. Place in Phosphomolybdic Acid for 2 minutes
3. Rinse in distilled water
4. Place in Picrosirius Red Stain for 1 hour. Save solution in original container when finished.
5. Place in .1 N Hydrochloride Acid for 2 minutes

Note: Pour used acid in labeled waste container

6. Place in 70% Ethanol for 45 seconds
7. Dehydrate by using program 2 on the autostainer
8. Mount with mounting media

OIL RED O

Purpose:

The demonstration of neutral lipids in frozen tissue sections. Fat occurring in an abnormal place such as fatty emboli that may develop after either a bone fracture or an injury that crushes a fatty body area may be demonstrated. The fat stain may verify that the emboli caused death. Degenerating material containing fat, such as cell membranes or myelin, may coalesce into fat droplets that are demonstratable with fat stains, and tumors arising from fat cells (liposarcomas) can be differentiated from other types of tumors.

Principle:

Staining with oil soluble dyes is based on the greater solubility of the dye in the lipoid substances than in the usual hydroalcoholic dye solvents. This is a physical method of staining and the dye used must (1) be more soluble in the tissue lipid than in the solvent in which it is dissolved, (2) not be water soluble, (3) be strongly colored, and (4) act with tissue constituents only by solution. The solvent used is critical, with isopropanol removing a minimal amount of lipid and propylene glycol not extracting any lipid.

Notes:

Lipids are removed by any fixative or solution containing alcohol or organic solvents, so sections cannot be processed for paraffin embedding. Frozen sections are most frequently used, but the use of water soluble wax for embedding will allow the demonstration of fat.

Free-floating sections stain more readily; however free-floating sections are difficult to obtain with the cryostat, and sections mounted on slides may be used. The staining time may need to be adjusted. Sections that have been fixed previously also may tend to loosen from the slides.

To improve the microtomy of frozen sections, formalin fixed tissues may be infiltrated with 30% sucrose solution before freezing.

Aqueous mounting media must be used, because the organic solvent present in synthetic resinous media will dissolve the fat.

The fat in the section is relatively liquid and mobile, so care should be taken that no pressure is placed on the coverglass or the fat may be displaced. If air bubbles are present in the section, remove the coverslip by soaking the slide in warm water. If glycerin jelly is used for mounting, it should not be overheated because this may melt the fat and also displace it.

OIL RED O

Reagents:

Oil Red O Stock Solution

Oil Red O - 2.5g

Isopropanol, 98% - 500mL

Mix well.

Oil Red O Working Solution

Oil Red O Stock Solution – 24mL

Distilled Water – 16mL

Mix well and let stand for ten minutes. Filter. The filtrate can be used for several hours.

Procedure:

1. Cut frozen sections, fix in 40% formaldehyde for 1 minute, and wash well in tap water; blot off excess water; if the tissue has been previously fixed, there is no need to refix.
2. Stain sections in oil red O for 10 minutes
3. Wash sections in tap water
4. Stain for 1 minute in Harris hematoxylin containing acetic acid (glacial acetic acid, 2mL/hematoxylin, 48mL)
5. Wash sections in tap water
6. Blue in ammonia water (or bluing)
7. Wash in tap water
8. Mount sections with an aqueous mounting medium
9. Seal the edges of the coverslip with fingernail polish

DAKO – Envision+ Dual Link System-HRP

- Envision+ Dual Link System-HRP has been optimally diluted for use with primary antibodies from mouse and rabbit in immunohistochemical procedures based on the labelled Polymer method.

- The Envision+ Dual Link System-HRP is a two-step IHC staining technique. This system is based on an HRP labelled polymer which is conjugated with secondary antibodies. The labelled polymer does not contain avid or biotin. Consequently, nonspecific staining resulting from endogenous avidin-biotin activity in liver, kidney, lymphoid, and cryostat sections is eliminated or significantly reduced.

- Kit is stored at 4C. All steps should take place at room temperature. Do not allow tissue sections to dry during the staining procedure.

Principle: Any endogenous peroxidase activity is quenched by incubating the specimen with an endogenous blocking reagent. The specimen is then incubated with a diluted rabbit primary antibody, followed by incubation with the labeled polymer, using two sequential 30-minute incubations. Staining is completed by a 5-10 minute incubation with a substrate-chromogen.

Description: Ki-67 is a nuclear protein, which is expressed in proliferating cells. Ki-67 is preferentially expressed during late G1-, S-, M-, and G2 phases of the cell cycle, while cells in the G0 (quiescent) phases are negative for this protein.

Positive Controls: Spleen, Tonsil, Breast

Labeled polymer: Peroxidase labelled polymer conjugated to goat anti-rabbit immunoglobulins in Tris-HCl buffer containing stabilizing protein and an anti-microbial agent.

Dako Antibody Diluent: intended for the preparation of primary and secondary antibody dilutions and negative control reagents for use in immunohistochemical (IHC) staining procedures.

Protocol:

1. Deparaffinization and Antigen Retrieval
    a. Make 1.5L 1X Antigen Retrieval Buffer from stock 100X Citrate Buffer (pH 6.0). (15mL Citrate + 1.5L Distilled Water)
    b. Pour this solution into one tank of the Lab Vision PT Module.
    c. Place slides in the slide holder and suspend the slide holder over the bath.
    d. Turn the module on and press “run” on the side that corresponds with the filled bath.
    e. Protocol takes about 2 hours including heating and cooling.
2. Endogenous Enzyme Block
    a. The following steps should be performed rapidly enough to prevent the tissue from drying out.
    b. Once module is cooled down, remove slides from slide holder.
    c. Tap off excess buffer onto paper towels and wipe the area around the tissue specimen with a kimwipe.
    d. Use a Pap pen to draw a circle around the tissue.

    e. Place slides on a staining tray and apply Dual Endogenous Enzyme Block dropwise enough to cover the specimen without overflowing the circle.
    f. Incubate for 10 minutes.
    g. Remove slides from staining tray and tap off the Enzyme Block onto paper towels.
    h. Place slides upright in PBS for 30 seconds.
3. Primary Antibody or Negative Control Reagent
    a. Remove the slides from PBS and tap off the excess and wipe the slides as before.
    b. Briefly spin down primary antibody (Ki-67, rabbit monoclonal, which has been diluted 1:200 in antibody diluent) or negative control reagent.
    c. Using a transfer pipette, apply enough diluted primary antibody or negative control reagent to cover the specimen.
    d. Incubate for 30 minutes.
    e. Tap off excess solution and place in PBS for 5 minutes.
    f. Repeat for a total of 2 washes.
4. Labelled Polymer-HRP
    a. Tap off excess and wipe slides as before.
    b. Apply labeled polymer dropwise to cover specimen.
    c. Incubate for 30 minutes.
    d. Tap off excess and place in PBS for 5 minutes.
    e. Repeat for a total of 2 washes.
    f. While waiting, add 1 mL DAB+ Substrate Buffer to an Eppendorf tube, then add 1 drop of chromogen.
    g. Invert the mixture several times to mix.
    h. Fill another tube with DI water.
5. Substrate-Chromogen
    a. Tap off excess buffer and wipe slides as before.
    b. Using a transfer pipette, apply enough substrate-chromogen solution to cover specimen.
    c. Incubate for 10 minutes.
    d. Using a transfer pipette, remove the solution from the slide and deposit it in the hazardous waste container designated for chromogen waste.
    e. Using the same transfer pipette, to move any unused substrate-chromogen solution to the chromogen waste container, then move the DI water from the Eppendorf tube to the tube that contained the substrate-chromogen solution. Close the tube, invert, and then pour into the chromogen waste container.
    f. Place slide upright in DI water for 1-2 minutes.
6. Hematoxylin Counterstain
    a. Remove slide from DI water and promptly transfer to autostainer,
    b. Use program #14 on autostainer and load the samples.
    c. Mount with mounting media and a cover slip.