Applied Histopathology

Prepared By:

Prof. Eman Abdel-Salam Ibrahim

Professor of Pathology

Faculty of Medicine

Ain Shams University

Second Year

2018/2019

Acknowledgments

This two-year curriculum was developed through a participatory and collaborative approach between the Academic faculty staff affiliated to Egyptian Universities as Alexandria University, Ain Shams University, Cairo University , Mansoura University, Al-Azhar University, Tanta University, Beni Souef University , Port Said University, Suez Canal University and MTI University and the Ministry of Health and Population(General Directorate of Technical Health Education (THE). The design of this course draws on rich discussions through workshops. The outcome of the workshop was course specification with Indented learning outcomes and the course contents, which served as a guide to the initial design.

We would like to thank **Prof.Sabah Al- Sharkawi** the General Coordinator of General Directorate of Technical Health Education, **Dr. Azza Dosoky** the Head of Central Administration of HR Development, **Dr. Seada Farghly** the General Director of THE and all share persons working at General Administration of the THE for their time and critical feedback during the development of this course.

Special thanks to the **Minister of Health and Population Dr. Hala Zayed and Former Minister of Health Prof. Ahmed Emad Edin Rady** for their decision to recognize and professionalize health education by issuing a decree to develop and strengthen the technical health education curriculum for pre-service training within the technical health institutes.

توصيف مقرر دراسى

1-بيانات المقرر		
الرمز الكودى:	**اسم المقرر** Applied histopathology : **تحاليل الانسجة التطبيقيه**	**الفرقة /المستوى:**
التخصص:	**عدد الوحدات الدراسية : نظري 2** **عملي 3**	

2- هدف المقرر:	This course aims at equip students with knowledge and skills about applied histopathology to enable students training on the different methods used in histopathology laboratories for the preparation and staining of different samples.
3-المستهدف من تدريس المقرر:	
ا. المعلومات والمفاهيم:	1 – Identify how to handle different tissue samples in tissue analysis laboratories 2 –Understand the methods of chemical preparation of different types of tissues, fluids and smears. 3 – Describe the method of routine staining of different samples on glass slides. 4 – Describe the methods of different special stains of samples on glass slides. 5 - Identify the steps of immunohistochemistry. 6 – Explain the preparation and staining of different samples in electron microscope laboratory.
ب- المهارات الذهنية:	1. Interpret the academic material 2. Compare different techniques of chemical preparation. 3- Differentiate different special staining techniques. 4- Differentiate immunohistochemical staining techniques. 5- analyze errors that lead to negative output
ج- المهارات المهنية الخاصة بالمقرر:	1 – Apply different ways of preparation of tissues, fluids and smears. 2 – Demonstrate the routine staining of sample glass slides. 3 - Demonstrate the different special staining techniques of sample glass slides. 4 – Apply the immunohistochemistry techniques. 5 –Demonstrate the preparation and staining of different samples in electron microscope laboratory. 6 – Apply the repair of different preparation artifacts. 7- Use different senses to master the work
د- المهارات العامة:	1 – Able to deal with the audience with elegance and interest. 2 – Work in caution and proficiency with different chemical reagents. 3 – Able to maintain professional rules to ensure the quality control of different techniques. 4 - Able to acquire new information and new experiences to cope with the development of the field of work.
4-محتوى المقرر:	1- Introduction to basic pathology 2- The basics of preparation techniques of different specimens -Different instruments and chemicals used in the technique -Different tissues

- Cytology and frozen sections + practical section 3- The basics of routine staining techniques -Instruments -Technique + practical section 4- The basics of some common required special stains + practical section 5- The basics of immunohistochemistry -Instruments - Technique + practical section 6- The basics of preparation and staining techniques in electron microscopy laboratories -Instruments -Techniques + practical section 7- The pitfalls in preparations and limitations 8- Revision	

1- Lectures using power point presentations 2- Practical session with availability of all required chemicals and equipment for practicing. 3- Field visits to different histopathology and electron microscopy labs. 4- Illustrative videos for different techniques. 5- Hand-outs to simplify of scientific material. 6- External readings of specialized books 7- Training to answer model question exercises.	5-أساليب التعليم والتعلم
1- Remedial lectures. 2- Remedial practicing lessons. 3- Simplified sketches for different techniques. 4- Training to answer model question exercises.	6-أساليب التعليم والتعلم للطلاب ذوى القدرات المحدودة
	7- تقويم الطلاب :
a. Class work: 1. Quiz 2. Midterm 3. Clinical skills b. Final exam written exam	أ- الأساليب المستخدمة
Class work: Quiz I (5th week) Midterm theoretical (7th week) Final exam written exam (15th week)	ب- التوقيت

ج- توزيع الدرجات	Quiz : 3 mark Midterm: 5 marks Attendance 2 marks Clinical: 10 marks Final written exam 80 marks. Total percentage 100 mark
: قائمة الكتب الدراسية والمراجع	
أ- مذكرات	Handouts for the lectures and practical sections

ب- كتب ملزمة	Applied histopathology, 1st edition , 2018. Ibrahim EA
ج- كتب مقترحة	1-Bancroft JD, Stevens A: Theory and Practice of Histological Techniques. 4th Ed., Churchill-Livingstone, New York, 1995 2- Luna LG: Histopathologic Methods and Color Atla s of Special Stains and Tissue Artifacts.American HistoLabs, Inc., Gaithersburg, MD, 1992
د- دوريات علمية أو نشرات ..... الخ	1-https://www.urmc.rochester.edu/urmc-labs/pathology/stainsmanual/index.html?MassonsTrichromeStain1 SPECIAL STAINS;

Contents

Course Description

This course aims at equip students with knowledge and skills about applied histopathology to enable students training on the different methods used in histopathology laboratories for the preparation and staining of different samples.

Core Knowledge

By the end of this course, students should be able to:

- Identify how to handle different tissue samples in tissue analysis laboratorie
- Explain the methods of chemical preparation of different types of tissues, fluids and smears.
- Describe the method of routine staining of different samples on glass slides.
- Describe the methods of different special stains of samples on glass slides.
- Identify the steps of immunohistochemistry.
- Explain the preparation and staining of different samples in scanning electron microscope laboratory.
- Recognize the preparation artifacts and limitations and how to repair them.

Core Skills

By the end of this course, students should be able to:

- Apply different ways of preparation of tissues, fluids and smears.
- Demonstrate the routine staining of sample glass slides. Demonstrate the different special staining techniques of sample glass slides.
- Apply the immunohistochemistry techniques.
- Demonstrate the preparation and staining of different samples in scanning electron microscope laboratory.
- Apply the repair of different preparation artifacts.
- Use different senses to master the work

Course Overview

		Methods of Teaching/Training with NumberofTotalHoursperTopic				
ID	**Topics**	**Interactive Lecture (2 h)**	**Field Work**	**Class Assignments**	**Research**	**Lab (2 h)**
1	**Introduction to basic pathology**	2				4
2	The basics of preparation techniques of different specimens.	4				6
3	The basics of routine staining techniques.	4				6
4	The basics of some common required special stains	6				8
5	The basics of immunohistochemistry	4				6
6	The basics of preparation and staining techniques in electron microscopy	4				6
	TOTAL HOURS (60)	**24**				**36**

1 Adapted from: Dimitra Project. (2011). *Communicating gender for rural development*. Available from http://www.fao.org/ docrep/014/am319e/am319e00.pdf

Chapter 1
Introduction to basic general pathology

Objectives

1- Define the term of histopathology
2- Identify different terms used to describe a pathological condition
3- Identify the different lesions encountered in histopathology
4- Describe the light microscope and identify its function
5- Enumerate other types of microscopes and define their functions

Overview of important definitions

Histopathology is the science concerned with the microscopic tissue changes characteristic of a disease. It serves as the bridge between the basic sciences and clinical medicine, and it is the scientific foundation for all medicine.

Cytopathology; is the science concerned with the cellular microscopic changes characteristic of disease.

The cell: is the smallest unit of a living body

Tissue: A large mass of similar cells that make up a part of an organism and perform a specific function.

Organ: is made up of different tissues. Eg.. heart (is made of muscular, vascular and connective tissues)

- The four basic tissues of the body are:

- Epithelium
- Connective tissue
- Muscle
- Nervous tissue

All of the organs of the body are composed of varying proportions of the four basic tissues, and each of the four basic tissues consists of cells and extracellular matrices.

-Histopathological examination is used to provides diagnostic information that is important for the diagnosis of the disease to determine treatment plan.

Tissue for study is obtained from
1-Biosy: Obtained during life either by open surgery (Excisional or incisional) or via endoscopy.
2-Autopsy: Obtained from the body after death.

Term used to describe pathological condition

1-**Morphology:** are characteristic changes in tissues and cells produced by disease in the living body, it includes Gross and microscopic features.

2-**Gross pathology**: is the description of pathologic changes of the disease by naked eye examination.
a) **Ulcer** is the loss of the surface covering epithelium.
b) **Nodule** is a small, rounded, irregular, distinct lump.
c) **Cavity** is an enclosed space filled with a content and separated from the surroundings by a wall.

3- **Microscopic pathology** is the description of pathologic changes of the cells or tissues by using microscopic tools.

Different lesions encountered in histopathology

1- **Congenital lesions:** is a medical condition that is present at or before birth. These conditions, also referred to as birth defects, can be acquired during the fetal stage of development or from the genetic make-up of the parents.
2- **Inflammatory lesions:** It is a vital process by which living tissues react to injury. It is done mainly to eliminate the causative agent and necrotic tissue in order to prepare tissue for the process of healing.

   a- acute -Suppurative (with pus) - Non suppurative (without pus)
   b- chronic
3- **Neoplastic lesions:** A neoplasm or a tumor is an abnormal mass of tissue, the growth of which exceeds and is uncoordinated with that of the normal tissues and PERSISTS after the cessation of the stimuli which may evoke this change.
   a- Benign
   b- Malignant -Carcinoma and - Sarcoma

Diagnostic Tools used in Histopathology

1-Ligt Microscopy (LM) (figure 1) is used for examination of tissue after staining by Hematoxylin & Eosin (H&E), special stains or immunohistochemical stains.

The microscope consists of a compound optical system (the objective lens and the ocular lens); a movable specimen support (the mechanical stage); an illumination system (the lamp and the condenser lens with its iris diaphragm). All the systems are attached to the microscope stand consisting of the base and arm.
The microscopes used in the course have a binocular head, which may be rotated after loosening its clamping screw. The topmost elements in the optical system are the eyepieces or ocular lenses. The inter-pupillary distance may be varied. One of the eyepieces may have a pointer, and note that one (or both) eyepiece(s) may be focused separately to compensate for dioptric differences between your eyes.
The revolving nosepiece is the inclined, circular metal plate to which the objective lenses, usually four, are attached. The objective lenses usually provide 4x, 10x, 40x and 100x magnification. The final magnification is the product of the magnification of the ocular and objective lenses. A slide is placed on the mechanical stage and is moved by rotating the stage control knobs. The lamp is an integral part of the base.
The fine and course focus controls are mounted coaxially on the stand just above the base. The arm connects the base to the binocular head-revolving nosepiece assembly.
The component of the illumination system immediately below the stage is the condenser
The height of the condenser may be varied to give a bright, evenly illuminated field. Generally, the

condenser is used in its highest position or just slightly lower. A lever projects from the condenser and it is used to vary the opening of the condenser (or iris) diaphragm. For work with the scanning (4x) and low-power (10x) objectives, the condenser diaphragm should be wide open. For work with the high-dry (40x) and oil-immersion objectives (100x), however, the diaphragm should be closed slowly while looking at a sharply focused section until the level of illumination is just slightly reduced. This is the setting of the condenser diaphragm for optimum contrast and resolution. (From a theoretical point of view this is not quite correct. The diaphragm should be adjusted for each magnification. In most instances, however, it is much less critical at the lower magnifications). In examining a slide with the light microscope, the following sequence of steps should be used:

1) Place the slide on the stage and examine it with the scanning objective (4x). Scan the entire section. Often tissue and organ identification can be made at this magnification.
Select an area or areas for study at higher magnification.
2) Rotate the revolving nosepiece to place the lower-power objective (10x) in the optical axis. When turning the nosepiece, grasp the nosepiece itself or the part of the objective adjacent to the nosepiece to avoid excess stress on the objective.
3) Proceed to the next step in magnification, if necessary, which is high dry (40x). Adjust the condenser.
4) For some specimens, especially blood and cellular organelles, you may want to use the highest magnification, which is oil immersion (100x). The following procedure must be used when working with the oil immersion lens:

a) Focus carefully on a selected area with the high-dry objective.
b) Swing the high-dry objective out of the light path and allow the nosepiece to remain in an intermediate position between the high-dry and the oil-immersion objectives.
c) Place a drop of immersion oil (available in the bookstore) on the slide in the appropriate region to be studied
d) Swing the oil-immersion. objective into position. The distance between the front element of the objective and the surface of the slide will be about 1.0mm, and the oil will form a bridge between the slide and the objective. The area to be examined should be within the field and should require only slight refocusing. You may need to readjust the condenser.

When finished using the oil-immersion objective, both the objective and the slide must be wiped with lens paper (available in the bookstore). If oil is allowed to dry on the high-dry or oil-immersion objective, the optical performance of the instrument will be severely reduced. The dried oil must be removed with lens paper.

Be careful not to move the high-dry objective into position while oil is on the slide. If this is done by mistake, the high-dry objective must be cleaned by wiping the front element with lens paper.

Slides should be cleaned with lens paper or tissue to remove fingerprints, oil or dirt, if the slide cannot be cleaned with a dry tissue, use alcohol.

A

B

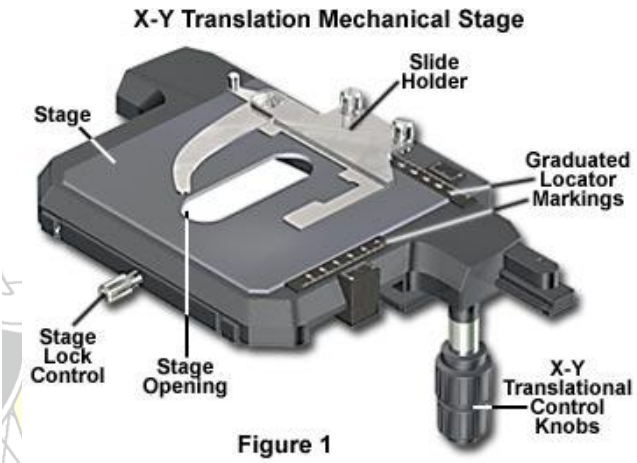
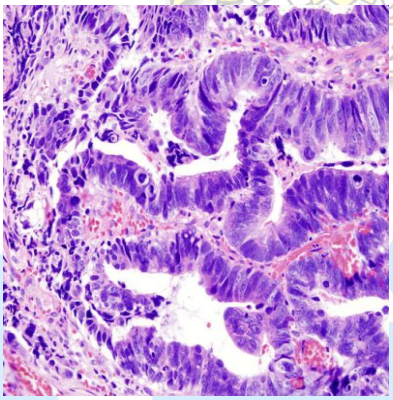


Figure 1

C



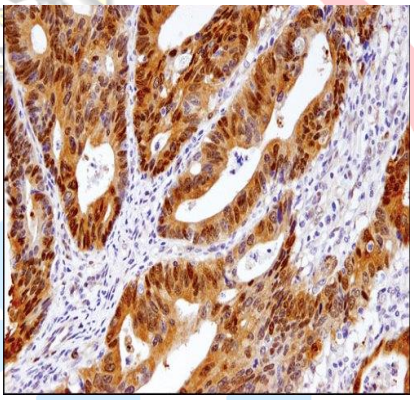


Figure (1): A; The light microscope.
B; The mechanical stage
C; Stained slides seen under the microscope. The left slide is stained with hematoxylin (the violet) and eosin (the pink). The right slide is stained by immunohistochemistry and sows positive results (the brown stain) with a background counterstain with hematoxylin (The blue).

2-**Electron Microscopy (EM)** is used for examination of cellular organelles by a magnification up to x500,000. It has a role in diagnosis of renal diseases. (figure 2 A & B)

A

B

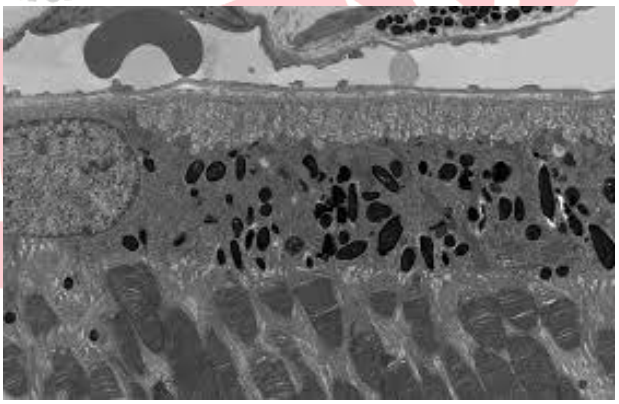


Figure (2) A; shows the electron microscope B; shows the produced black and white images.

3- **fluorescent microscopy:** looks like the light microscope but it uses laser as a source of illumination: used to visualize specimen slides stained by immunohistochemistry using fluorescent kits. (figure 3)

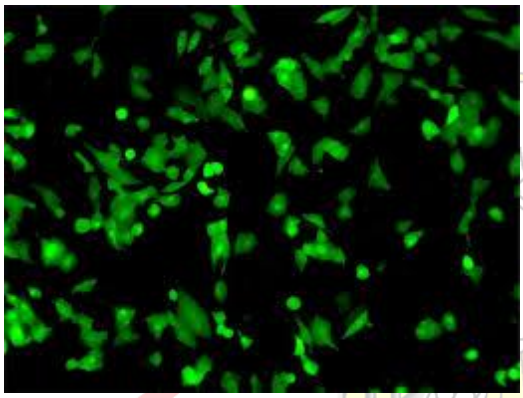


Figure (3): A specimen on glass slide visualized under fluorescent microscope.

4-**Polarized light microscope**: It looks like the light microscope, but it uses polarized light as a source of illumination. This type of **light** is used to improve image quality when examining birefringent (doubly-refracting), anisotropic materials (figure 4). Anisotropic substances are "direction-dependent" – that is, they **do** not behave the same way in all directions.

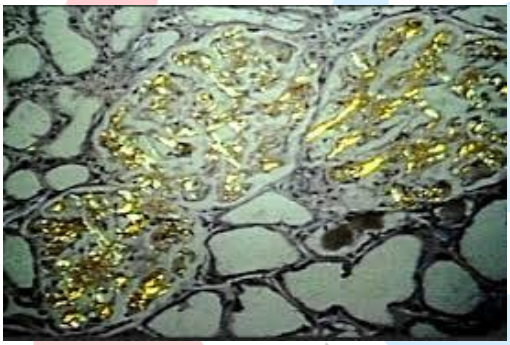


Figure (4): A specimen on glass slide visualized under polarized light microscope.

Chapter 2

The basics of preparation techniques of different specimens

Objectives

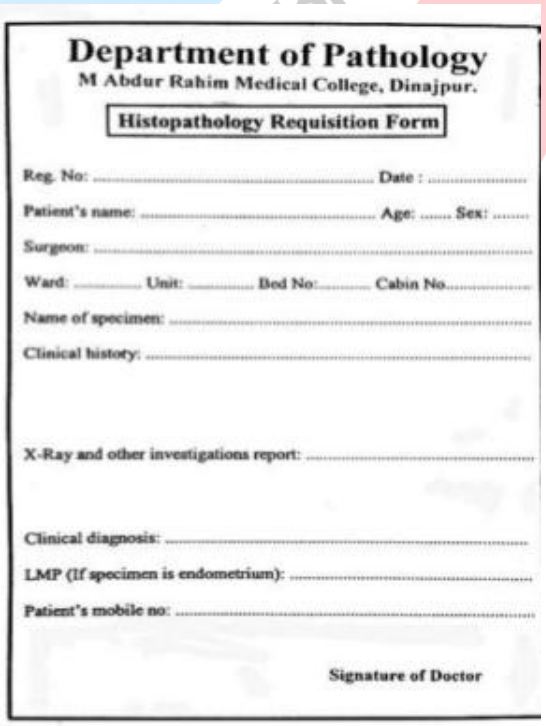
1- Enumerate the requirements to accept a specimen.
2- Describe the preparation procedure of different tissues.
3- Identify different instruments used in the technique.
4- Identify different chemicals used in the technique.
5- Describe the preparation procedure of Cytology and frozen sections**.**

Requirements to accept a specimen sent to a histopathology lab.

1- A Histopathology request.
2- Container
3- Fixative.

A histopathology Request (figure 5)**:**

a) One request for one patient.
b) All data should be filled properly.
c) If more than one specimen is received for the same patients, they should be labelled correctly (a,b,c) (right or left) and the containers should be labelled the same.



Department of Pathology
M Abdur Rahim Medical College, Dinajpur.
Histopathology Requisition Form

Reg. No: ..... Date : .....
Patient's name: ..... Age: ..... Sex: .....
Surgeon: .....
Ward: ..... Unit: ..... Bed No: ..... Cabin No. .....
Name of specimen: .....
Clinical history: .....
X-Ray and other investigations report: .....
Clinical diagnosis: .....
LMP (If specimen is endometrium): .....
Patient's mobile no: .....
Signature of Doctor

Figure (5): Histopathology request form.

Container

a) Plastic or glass jar
b) Label matching requisition slip (registration number, Full patient's name)
c) More specimen mark as A, B, C….

Fixative

Formalin

-!0% formalin (unbuffered) = (40% formalin 100 ml + 400 ml tap water).

-!0% neutral buffered formalin (NBF) = 40% formalin 100 ml + 400 ml tap water + Na hydrogen PO4 0.4gm + Di Na hydrogen PO4 0.65 gm in 7.2-7.4 PH)

Advantages:

a- Rapid penetration
b- Easy availability and cheap price.
c- Does not over-harden the tissues.
d- Fixes lipids for frozen sections.
e- Ideal for mailing.
f- NBF prevents formation of (acid formaldehyde hematin) formed from hemoglobin in acidic PH.

Disadvantages:

1- Irritant to nose, eyes and mucous membranes
2- Forming a precipitate of paraformaldehyde which can be prevented by adding 11-16% methanol
3- Formation of black formalin pigments (acid formaldehyde hematin).

Causes of rejection of specimen:

1- Specimen not in formalin.
2- Unlabeled or improperly labeled container.
3- Without requisition slip or incomplete requisition slip.

The preparation procedure of different tissues:

Fresh tissues are extremely fragile and subject to autolysis; so it must be fixed and chemically prepared for sectioning (sections thin enough to be translucent), staining and examination.

Loss of specimen is tragic for both the patient and the pathologist; so handling of all specimens with care and respect is mandatory.

Specimens should be labelled in the Lab. By ID numbers.

A) Fixation of Tissues: Good fixation is most important in the production of satisfactory results in histopathology.

Aims:

1- Should prevent autolysis or putrefaction of the cell
2- Should penetrate evenly and rapidly.
3- Should harden the tissues.
4- Increase the optical density.
5- Should not cause shrinkage or swelling of the cells.
6- Must not react with the receptor sites and thus must not interfere with the staining procedure.

It SHOUD NOT be used in

1- Frozen sections
2- Immunofluorescence
3- Cultures
4- Chromosome study
5- Electron microscope.
6- Flow-cytometry

There are two types:

a- Manual tissue processing.

b- Mechanical tissue processing (Done by automatic machine) (figure 6).

1. For routine stains, tissue is sectioned and drop-fixed in a 10% formalin solution. Fixative volume should be 10 times that of tissue on a weight per volume.
2. Due to the slow rate of diffusion of formalin (0.5 mm/ hour), tissue should be sectioned into 3 mm slices before transfer into formalin. This will ensure the best possible preservation of tissue and offers rapid uniform penetration and fixation of tissue within 3 hours.
3. Tissue should be fixed for a minimum 48 hours at room temperature.
4. After 48 hours of fixation, move tissue into 70% ethanol for long term storage.
5. Keep fixation conditions standard for a particular study in order to minimize variability.

The usual fixative for paraffin embedded tissues is neutral buffered formalin (NBF). Inadequately fixed tissues will become dehydrated during tissue processing, resulting in hard and brittle specimens.

A particular challenge for the histopathology is immunostaining fixed specimens. In many cases formaldehyde fixation will prevent recognition of epitopes by the primary antibody. Occasionally, "antigen retrieval" procedures will improve results but usually frozen sections are a better bet.

Decalcification of bone (optional)

After fixation, bone must be decalcified, or else it won't cut on the microtome:

- Immerse tissue cassette in 11% formic acid with a stir bar overnight in a fume hood.
- Rinse in running water for 30- 60 minutes (the smell should be gone).

Storage in 70% Ethanol

After adequate fixation tissues are transferred to 70% ethanol and may be stored at 4°C.

In summary: Tissue processing

Thickness	No more than 3 mm thick.
Area	20 mm × 30 mm.
Fixed tissue	Cut large organs into 3 mm slices and store in neutral buffered formalin for 48 hours. Select tissue from fixed areas, trim to size and re-fix until the evening. If the trimmed sample is visibly unfixed, re-fix for a further 24 hours.
Unfixed tissue	Slices of tissue should be *thoroughly* fixed before processing.
Times	All times in processing fluids for this schedule are for tissues 3 mm thick or less. Tissues thicker than that will require longer times.
Clearing agent	Xylene or another clearing agent that will clear tissues in similar times should be used.
Processing time	This schedule takes 12 hours, and processes overnight. On weekends tissues should be left in fixative until Sunday evening with a 48 hour delay.

Trim fixed tissues and keep in neutral buffered formalin (NBF) until ready to proceed. Put tissues in a labeled (usually with pencil, as solvents dissolve the ink) cassette.

Once fixed, tissue is processed as follows, using gentle agitation, usually on a tissue processor, as follows:

1. 70% ethanol for 1 hour.
2. 95% ethanol (95% ethanol/5% methanol) for 1 hour.
3. First absolute ethanol for 1 hour .
4. Second absolute ethanol 1½ hours .
5. Third absolute ethanol 1½ hours.
6. Fourth absolute ethanol 2 hour.
7. First clearing agent (Xylene or substitute) 1 hour.
8. Second First clearing agent (Xylene or substitute) 1 hour.

The tissue is ready for paraffin infiltration:

9. First wax (Paraplast X-tra) at 58°C for 1 hour.
10. Second wax (Paraplast X-tra) at 58°C 1 hour.

Figure (6); Histology tissue processor.

Spin vacuum tissue processor. -Automatic operating control

-Tissue basket relocating operating system in jars containing th

-Large touch screen.

B) Paraffin infiltration

In this procedure, tissue is dehydrated through a series of graded ethanol baths to displace the water, and then infiltrated with wax. The infiltrated tissues are then embedded into wax blocks. Once the tissue is embedded, it is stable for many years.

The most commonly used waxes for infiltration are the commercial ***paraffin waxes***. The wax is solid at room temperature but melts at temperatures up to about 65°C or 70°C. Paraffin wax can be purchased with melting points at different temperatures, the most common for histological use being about 56°C–58°C, at its melting point it tends to be slightly viscous, but this decreases as the temperature is increased. The traditional advice with paraffin wax is to use this about 2°C above its melting point. To decrease viscosity and improve infiltration of the tissue, technologists often increase the temperature to above 60°C or 65°C in practice to decrease viscosity.

In the schedule below, it is presumed that the working day is from 8:00 a.m. to 5:00 p.m. If other than that, appropriate adjustments should be made.

Due to the viscosity of molten paraffin wax, some form of gentle agitation is highly desirable. If the processor is to be run overnight it should be programmed to hold on the first ethanol bath and not finish until the next morning so the specimens do not sit in hot paraffin longer than the time indicated. If specimens are fresh they may incubate in formalin in the first stage on the machine. It is important to not keep the tissues in hot paraffin too long or else they become hard and brittle. Processed tissues can be stored in the cassettes at room temperature indefinitely.

C) Embedding tissues in paraffin blocks

Tissues processed into paraffin will have wax in the cassettes; in order to create smooth wax blocks, the wax first needs to be melted away placing the entire cassette in 58°C paraffin bath for 15 minutes. Turn the heat block on to melt the paraffin one hour before adding the tissue cassettes.

1. Open cassette to view tissue sample and choose a mold that best corresponds to the size of the tissue. A margin of at least 2 mm of paraffin surrounding all sides of the tissue gives best cutting support. Discard cassette lid.
2. Put small amount of molten paraffin in mold, dispensing from paraffin reservoir (paraffin wax dispenser) (figure 7).
3. Using warm forceps, transfer tissue into mold, placing cut side down, as it was placed in the cassette.
4. Transfer mold to cold plate, and gently press tissue flat. Paraffin will solidify in a thin layer which holds the tissue in position.
5. When the tissue is in the desired orientation add the labeled tissue cassette on top of the mold as a backing. Press firmly.
6. Hot paraffin is added to the mold from the paraffin dispenser. Be sure there is enough paraffin to cover the face of the plastic cassette.
7. If necessary, fill cassette with paraffin while cooling, keeping the mold full until solid.
8. Paraffin should solidify in 30 minutes. When the wax is completely cooled and hardened (30 minutes) the paraffin block can be easily popped out of the mold; the wax blocks should not stick. If the wax cracks or the tissues are not aligned well, simply melt them again and start over.

-The tissue and paraffin attached to the cassette has formed a block, which is ready for sectioning. Tissue blocks can be stored at room temperature for years.

Figure (7): Paraffin wax dispenser.

D) Sectioning tissues

Tissues are sectioned using a microtome (Figure 8). Turn on the water bath (figure 9) and check that the temp is 35-37ºC. Blocks to be sectioned are placed face down on an ice block or heat sink for 10 minutes. Place a fresh blade on the microtome; blades may be used to section up to 10 blocks, but replace if sectioning becomes problematic. Insert the block into the microtome chuck so the wax block faces the blade and is aligned in the vertical plane.

Set the dial to cut 10 µM sections to order to plane the block; once it is cutting smoothly, set to 5 µM sections . The blade should angeled at 5º. Face the block by cutting it down to the desired tissue plane and discard the paraffin ribbon. If the block is ribboning well then cut another four sections and pick them up with forceps or a fine paint brush and float them on the surface of the 37ºC water bath. Float the sections onto the surface of clean glass slides. If the block is not ribboning well then place it back on the ice block to cool off firm up the wax. If the specimens fragment when placed on the water bath then it may be too hot.

Place the slides with paraffin sections on the warming minutes (so the wax just starts to melt) to bond the overnight at room temperature.

Figure (8): Microtome aim to section the paraffin block at to 5 µM thickness.

Figure (9): Paraffin section mounting path; which allows the warm water held within to 'float' each section onto a slide. The wax ribbon will be slightly crinkled as they are cut and the bath helps to flatten this out. It is important to do this slide by slide from the ribbon, so that the slices can be chronologically identified. The bath should be kept at 45 degrees C throughout the mounting stage.

Figure (10): Histopathology lab oven

Cytopathology:
Is a branch of histopathology that study and diagnose diseases by evaluation of cellular changes that happen to the cells.

Types of cytology samples:

1- Exfoliative cytology: is the study of cells that have been shed or removed from the epithelial surface of various organs. (wash, smear, scraping and brushing)
2- Fine needle aspiration cytology: Is used to obtain material by needle from organs or lesions by needle aspiration. It is valuable in diagnosis of the breast, thyroid, lymph nodes and liver lesions.
3- Body fluids: like urine, pleural fluid, pericardial fluid, cerebrospinal fluid, synovial fluid and ascitic fluid can be studied for cytopathology.

Advantages of cytopathology:

1- A non-invasive procedure
2- Helps in faster reporting and guide the clinician
3- Relatively inexpensive
4- Has high population acceptance.
5- Facilitates cancer screening.

Factors affecting optimal cytological preparation:

1- Quality of the specimen: Too bloody, too thick, or has been left too long before being taken to the laboratory, the results will be less than optimal.
2- Fixative chosen to preserve the cellular details: If fixative not correct for the specimen or not applied quickly, the results again will be less than optimal.
3- The stains: If they are old, not properly prepared, or not optimized for time, the results again will be less than optimal.

Steps of preparation:

1- Evaluation: Bloody smears need additional treatment by "CytoLyt" to lyse The RBCs. Mucoid specimens need to be treated with "Mucolexx" to thin and break down the mucous for further preparation
2- Gross examination: Determine the volume, the colour and the appearance
3- Preparation of smears: It means spread of obtained material on glass slides. They are usually made directly from the aspirated fluid as rapid as possible to prevent dry artifacts on the cells.
   If delay is anticipated, the fluid is kept in the refrigerator for 24-48, or addition of ethanol 2:1. This will keep the sample up to 7 days.
   If too much fluid is obtained, centrifuge (figure 11) must be used for 5 minutes and the sediment is used to make the smear.
4- Fixation: The purpose is to preserve The cyto-morphologic characteristics of the cells.

Types of fixatives:

-Dry fixation: The slide dry by air quickly after the material is spread on the slide followed by hematological stain.

-Wet fixation: Ethanol 95% is the best. For transportation, we can use spray fixative of alcohol with wax.

-Liquid-based fixation: The sample is collected to vials containing methanol. It is suitable for transportation and can be stored up to 7 days.

-Lysing fixation for bloody smears: by ready to use preparation (CytoLyt, CtoRich Red) for bloody smears.

Figure (11): Centrifuge: Used in cytopathology.

Frozen Sections procedure: is a pathological laboratory procedure to perform rapid microscopic analysis of a specimen. It is used most often in oncological surgery. The technical name for this procedure is cryosection.

Procedure: The key instrument for cryosection is the cryostat (fig 12), which is essentially a microtome inside a freezer; it produces sections as thin as 1 micrometre. The surgical specimen is placed on a metal tissue disc which is then secured in a chuck and frozen rapidly to about –20 to –30 °C. The specimen is embedded in a gel like medium called OCT and consisting of poly ethylene glycol and polyvinyl alcohol; this compound is known by many names and when frozen has the same density as frozen tissue. At this temperature, most tissues become rock-hard. Usually a lower temperature is required for fat or lipid rich tissue. Subsequently it is cut frozen with the microtome portion of the cryostat, the section is picked up on a glass slide and stained (usually with hematoxylin and eosin, the H&E stain). The preparation of the sample is much more rapid than with traditional histology technique (around 10 minutes vs 16 hours). However, the technical quality of the sections is much lower.

Figure (12): Cryostat; a freezing and sectioning machine used in processing of frozen sections.

Chapter 3

The basic routine staining technique

Objectives

1-Identify types of routine staining techniques and advantages.
2-Identify different reagents uses and troubleshooting.
3-Explain the Rotation/Replacement of Solutions Guideline.
4-Describe General comments in preparing/using solutions.

Types of routine staining techniques:

The specimen on the microscope slide is a thin section (usually 5 micrometers) of the fixed tissue or organ. The section is stained by one or more dyes. Without staining the section would be nearly invisible with the microscope.

-There are two types of staining

1- Manual staining.

2-Automatic staining. It has a mechanical device (figure 13) which shifts the slides from one container to next after the specified time.

Figure (13); Automatic autostainers

Advantages:

a- It reduces the man power
b- It controls the timing of staining accurately.
c- Large number of slides can be stained simultaneously
d- Less reagents are used

I REAGENTS

A. **Clearants:** Xylene or Xylene Substitutes

1. **Use.** Clearants are used in two different stages in H&E staining.

a. Deparaffinization (removal of paraffin).

. (1) **Troubleshooting.** If paraffin is not totally removed from tissue sections, color intensity may be decreased or staining may be irregular(spotty) within the tissue section.

. (2) **Recommendations for deparaffinization:** Use three changes of xylene, 3 minutes each station. If an automatic stainer is used where there are limited numbers of staining vessels, use at least 2-3 changes of xylene irrespective of time. If using xylene substitutes, follow the directions provided by the manufacturer. Xylene substitutes are slower in action, and often require longer times or more stations than xylene.

b. Clearing – displacement of alcohol from the tissue sections with the clearant to assure miscibility when cover-slipping with xylene – toluene or other petroleum-based mounting media.

(1) **Troubleshooting**

a. Inadequate time in the xylene will allow anhydrous (100%) alcohol to remain within the tissue sections. The presence of alcohol may cause the eosin to bleed from the tissue section after cover-slipping.

b. Inadequate dehydration (removal of non-anhydrous (70%, 95% alcohol) from the tissue section will cause slides to be hazy or milky. Removal of non-anhydrous alcohol is not the role of the clearant; it is the role of anhydrous (100%) alcohol. The haziness/milkiness observed with the cover-slipped slide is usually caused by the mixing of water from the alcohol with the clearant.

(2) **Recommendations for clearing**: Use three changes of xylene, one minute minimum per station. If an automatic stainer is used where there is a limited number of staining vessels, use at least 2-3 changes of xylene irrespective of time. If using xylene substitutes, follow the directions provided by the manufacturer.

2. **Varieties.** The grade of xylene used should be known and carefully monitored. Lower grades (technical, industrial, engineering) often contain other petroleum products that can interfere with staining. The presence of these unwanted petroleum products can be easily detected by their extraneous odor.

B. Alcohols: ethanol, denatured ethanol, isopropyl alcohol

1. **Use.** Alcohols are used in two different stages in H&E staining.

a. Hydration – the introduction of water into the tissue section. This is done by passing the slides slowly through a series of decreasing concentrations of alcohols.

Troubleshooting. If the clearant is not displaced by the anhydrous alcohol, subsequently allowing carryover of the clearant into lower concentrations of alcohol, clearant droplets (oil droplets) will be visible in the lower alcohols and sometimes in the water. In extreme cases, the clearant may remain in the section and will interfere with hematoxylin staining.

(2) **Recommendations for hydration**: The first alcohol used after the clearant must be anhydrous (100%). To prevent the carryover of the clearant into lower alcohol concentrations, three changes of anhydrous alcohol are suggested, followed by alcohols of lower concentrations: 95% (1-2 changes), 70% (1 change), and sometimes 50% (1 change). Immersion times should be sufficient to assure the complete removal of the previous solutions. One minute is adequate for each station. If an automatic stainer is used where there are limited numbers of staining vessels, use at least 2-3 changes of anhydrous alcohol irrespective of time. To further save stations it is possible to go directly from 95% alcohol into water without adverse effects; however, graded series may be needed for delicate specimens or weakly adhered sections.

b. Dehydration – the removal of water from the tissue section.

(1) **Troubleshooting.** Increasing concentrations of alcohol after eosin staining are used to remove water from the tissue section. If all water molecules are not removed from the tissue section, proper clearing cannot be achieved. Inadequate removal of non-anhydrous (70%, 95%) alcohol prior to entering the clearant for cover-slipping will cause slides to be hazy or milky. The haziness is due to the mixing of the water in the alcohol with the clearant.

(2) **Recommendations for dehydration:** The use of 70% or 95% alcohol after eosin is dependent upon desired eosin intensity. With or without the use of 70% or 95% alcohols, three changes of anhydrous alcohol should be used, one minute each. If an automatic stainer is used where there are limited numbers of staining vessels, use at least 2-3 changes of anhydrous alcohol irrespective of time.

2. **Storage.** All alcohols have the capacity to absorb moisture from the air. Humidity can interfere with proper dehydration and clearing of the slides; therefore, it may be necessary to rotate anhydrous (100%) alcohols more frequently during humid conditions. Storage of anhydrous alcohol should be in containers that allow minimal exposure to air. With a change in temperature, the moisture in the air inside the container could condense into water droplets that would contaminate the alcohol. If anhydrous alcohol is purchased in 55 gallon drums, inlet bungs should be set up with a drying tube (especially with humid conditioned or when the drum is used over a long time period) that will remove the moisture from the air before it enters the drum.

C. **Hematoxylin:**

Hematoxylin is not a true basic dye. It is used with an intermediary, which recognizes anionic tissue components. Hematoxylin is nearly a specific stain for chromatin and it is therefore referred to as a "Basic" stain. It stains the nuclear network, chromosomes, etc., blue. It is a regressive stain and is extracted by very dilute acid or acid alcohol. It may be used after almost any fixative and is a permanent stain.

1. **Use.** Hematoxylin is used after de-paraffinization and hydration.

a. **Troubleshooting**

(1) Poor hematoxylin staining can be due to:

(a) Autolysis or poor fixation.

(b) Incomplete de-paraffinization

(c) Over-decalcification

(d) Inadequate staining time

(e) Destaining – too strong or excessive time

(f) Weak hematoxylin that has lost its potency with age or carryover of water.

(g) Contaminants in rinsing solutions.

(h) Thin sections

(i) Incorrect pH of the hematoxylin

(j) Inadequate removal of alcohol or insufficient pre-rinsing with water prior to staining with hematoxylin

(2) Excessive staining of hematoxylin can be due to:

(a). Drying of tissue section.

(b). Strong potency of hematoxylin from "topping up", change in formulations, or age

(c). Excessive staining times

(d). Excessive slide adhesive (e.g., albumin, gelatin)

(e). Destaining – too weak or inadequate time

(f). Thick section

(g). Prolonged exposure to heat

(3) Removal of metallic sheen. Except for Gill hematoxylins, all hematoxylins develop a surface metallic sheen upon standing or with use. If not removed, the metallic sheen will appear as a due precipitate on the slide. Therefore, all hematoxylins except Gill require daily filtering to remove the metallic sheen.

2. **Varieties.** There are numerous formulations of hematoxylin available. Individual preference of the pathologist or diagnostician must be a primary consideration. The active ingredient in hematoxylin solutions is hematein complexed with a metal ion (eg, aluminum, iron, tungsten). Aluminum is the most commonly used. If aluminum is used, the hematoxylin solution will stain blue; if iron is used hematoxylin will stain black or blue-black. The ratio of metal to hematein may also influence the color.

Hematein is formed by oxidizing hematoxylin. Oxidizing agents used are air, sodium iodate, mercuric oxide, and potassium permanganate. Hematoxylin formulae that use mercuric acid as an oxidizer warrant special disposal considerations because of the mercury. Over-oxidation will cause poor staining and can occur during the initial manufacturing or with aging of the hematoxylin.

The mode of hematoxylin staining can be progressive or regressive. Regressive staining overstains the tissue and then decolorizes the tissue with an acid solution. Progressive staining stains to desired intensity without initially over-staining. Progressive hematoxylins may be used in a regressive method.

Progressive Hematoxylins: Mayer's

Delafield's

Gill's

Harris'

Regressive Hematoxylins: Delafield's

Harris'

Ehrlich's

These formulations provide a variety of hues, potencies, and staining patterns. Gill hematoxylins have three formulations (I, II, and III). Gill I has a strength which stains the delicate chromatin pattern in cytological preparations. Gill II and III are used in tissue staining. Gill III, because of its added strength is often used in frozen sections.

D. **Bluing Reagent:** Ammonia solutions, tap water, Scott's solution, and lithium carbonate solutions.

1. **Use.** Bluing reagents change the reddish – purple hematoxylin to a blue or purple blue color. It is a pH dependent reaction and occurs in an alkaline solution.

a. **Troubleshooting.** Reddish color of a stained section is due to inadequate bluing. Bluing reagents should have a pH of approximately 8. It is not possible to over-blue a section. The bluing reagent can only blue the amount of hematoxylin in the tissue. If the section is too blue, there is too much hematoxylin in the section. Sections may fall off it left too long in a harsh bluing agent, such as an ammonia solution.

2. **Recommendations for bluing:** With dilute aqueous solutions (e.g. water, Scott's solution) no more than one minute is required. More potent reagents (ammonia solutions) often require less time. Since the bluing reaction has a specific end point (i.e. it cannot be over-done), the timing of the bluing step can easily be determined visually when the tissues turn "blue".

3. **Varieties:** There are numerous formulations of bluing reagents available. The active ingredient in any bluing reagent is the alkaline material – either ammonia or alkaline salts such as lithium or magnesium carbonate. The choice of whether to use tap water as a bluing reagent will depend upon the quality of the local tap water (See Water). Formulations of alcoholic bluing reagents work faster and are therefore better on linear stainers and for use by those individuals who use quick dip methods.

E. **Hematoxylin differentiator:** acetic acid, hydrochloric acid

1. **Use.** In the regressive hematoxylin staining method one purposely overstains the tissue sections, the removes the excess stain by using an acid rinse. An acid rinse is also used to remove non-specific hematoxylin staining (e.g. staining of the glass slide) with progressive or regressive stains.

2. **Troubleshooting:**

a. <u>De-staining will be excessive if:</u>

(1) Concentration of acid is too great

(2) Concentration of water is too great as with alcohol-based differentiators

(3) Destaining time is excessive

(4) Decolorization agent is not completely removed with post water rinse

b. <u>De-staining will not be sufficient if:</u>

(1) Concentration of acid is sufficient

(2) Concentration of alcohol is too great as with alcohol-based differentiators

(3) Destaining time is inadequate

(4) There is an excess of albumin/gelatin on the slide

3. **Recommendations for hematoxylin differentiator:** A water-based acid rinse acts quickly to de-stain the tissue section, making timing critical to the second. To allow more control and reproductively, alcohol-based acid solutions are used which slow down the rate of decolorizing. Therefore, most acid rinses are made up in 70% - 95% alcohol. The concentration and type of acid (hydrochloric or acetic) used will determine the timing in the acid alcohol solutions. Acetic acid is weaker than hydrochloric acid; thus, its concentration must be higher. If purchasing commercially available hematoxylin, check manufacturer's directions for the recommended acid to use in the differentiation step.

4. **Varieties:** The type of acid rinse used will be determined partially by the method of staining. Hand staining, with the quickness of the hand, can handle the stronger hydrochloric acid and more aqueous-based solutions. In automatic staining it is necessary to use alcohol-based acid solutions, and/or weaker acids (e.g. acetic acid) or weaker concentrations of acids.

F. **Water:** Tap water, distilled/deionized water

1. **Use:** Water is used in H&E procedures in critical steps of rinsing before and after hematoxylin staining, as a possible bluing reagent, and in diluting alcohols.

2. **Troubleshooting**

a. Tap water often contains elements that can de-stain hematoxylin. Iron acts as mordant, sulfur tends to acidify water, and chlorine is a bleach.

b. Tap water may not be alkaline enough (pH greater than or equal to 8) to act as a bluing reagent. The pH of tap water can fluctuate daily and seasonally.

c. Water may contain particulate material that may adhere to the tissue sections.

3. **Recommendations for water:** Run water from the tap for a minute, then check its pH to determine if it is alkaline enough to be a bluing reagent. If iron or sulfur is a common element in the tap water, it is best to use deionized or distilled water for all steps after staining. Distilled/deionized water generally produces consistent results.

G. **Eosin:**

Eosin, eosin-phloxine

1. **Use:** Eosin in the H&E procedure is referred to as a counterstain. It stains nearly everything that hematoxylin will not stain. When applied correctly, eosin produces three different hues which can be used to differentiate various tissue elements; red blood cells stain dark reddish orange, collagen stains a lighter pastel pink, and smooth muscle stains bright pink.

2. **Troubleshooting**

a. <u>Weak staining of eosin can be due to:</u>

(1) pH of the eosin – too high

(2) Alcohol rinse after the eosin – too long or too aqueous

(3) Contaminant in the alcohol rinse

(4) Deteriorating eosin due to excessive carryover

(5) Thin sections

(6) Inadequate staining time

b. <u>Excessive staining of eosin can be due to:</u>

(1) Stronger dye solution due3 to excessive evaporation

(2) Use of isopropyl alcohol as the rinsing agent

(3) Thick sections

(4) Excessive staining times

(5) pH of the eosin – too low

c. Undifferentiated eosin staining (one color) can be due to:

(1) Poor fixation

(2) Overstaining due to:

(a) Aqueous eosin stain

(b) Excessive staining time

(c) Stain too strong

3. **Recommendations for eosin staining:** Generally, there are only three significant variables that influence proper eosin staining: eosin staining times, concentration of alcohols following the stain, and time in these alcohols. Eosin is very soluble in water. Therefore, the more water there is in the alcohol following the eosin, the more eosin there will be removed from the tissue. If isopropyl alcohol must be used due to cost or the unavailability of ethanol, staining protocol must consider the limitations of isopropyl alcohol as a rinsing alcohol. Anhydrous isopropyl alcohol has a poor solubility for eosin, and therefore does not rinse off the excess eosin. Diluted isopropyl alcohol (70%, 95%) can remove excess stain.

4. **Varieties:** There are numerous formulations of eosin available. Solutions can be either alcoholic or aqueous. Phloxine, picric acid, and/or orange G may be added to eosin solutions to give stronger red or orange tones.

II Rotation/Replacement of Solutions Guideline

A. **All staining solutions or reagents have a definite useful life**. Use the appearance of the symptoms under the sections Troubleshooting to assist your lab in determining rotation or replacement of solution schedules. Rotation/replacement schedules vary among labs because of work volume, type of solutions used, and mode of staining (automatic or manual).

B. **Wash and rinse thoroughly each staining vessel at the time of rotation**. Inadequate washing or rinsing can leave residues that can alter the chemical nature of new solutions placed in the vessel. Multiple water rinsing is mandatory when using detergents and acid rinses.

C. **Alcohol dehydration/clearant rotation**. A general rule is to rotate the final anhydrous alcohol when it acquires a pink cast from eosin carryover. Rotate the adjacent clearants at the same time. Whenever the appearance of solution looks unusual (e.g. milky alcohols, appearance of "beads" of water or clearant), discard and replace that solution immediately.

D. **Hematoxylin/Eosin Replacement**. Replacement schedules vary among labs because of work volume, type of solutions used, and mode of staining (automatic vs. manual). The information provided in the stain's Troubleshooting section along with microscopic examination of the control

slide will help determine a regular replacement schedule for one's lab.

E. **Bluing Reagent**. If tap water is used, it should be changed with each rack/set of slides. Prepared bluing reagents should be changed at least daily.

III. Record Keeping

A. Daily activities.

1. Record on the daily staining log the following information. A staining log should be kept for each staining setup or automatic stainer.

a. The brand and lot number of each stain used.

b. The pH of the eosin and hematoxylin.

c. Filtering of the hematoxylin.

d. Temperature and pH of water.

e. The rotation/discarding/replacement of reagents.

f. With multiple automatic stainers, record which slides are stained on which stainer.

2. Run an H&E control slide and document results. (For thorough records, a control slide should be run in each slide rack)

3. Provide the pathologist with a slide evaluation checklist to document the quality of the slides.

B. Control of purchased materials

1. Have written specifications for reagents.

a. Denatured alcohol should include the denaturants and their percentages.

b. Generic reagents (xylene, acids, bases, salts)

(1) Chemical formulation with any waters of hydration

(2) Grades (e.g. Reagent, Technical, USP, etc)

(3) CAS number

c. Dyes

(1) C1 number

(2) Certifications by the Biological Stain Commission

2. Mark reagents in conflict with expiration date, use on a first used basis. Date each reagent upon

to indicate the date of receipt. High-light expiration date. Unless opening.

IV. General comments in preparing/using solutions.

A. Determining optimal staining **times**: The length of staining time will be dependent upon the desired intensity. Work closely with the pathologist or diagnostician to determine the preferred staining time. During trials to determine staining times, use all fresh solutions in the staining sequence. Use serial sections or control slides during trial staining. Recommended staining times can be obtained from textbook references or manufacturer's directions. When trying to correct any fault in the stained slide, make only one change at a time in the staining schedule. Record each change and the results before making subsequent changes.

B. **Use all solutions at room temperature.**

C. **Have complete written specifications for all reagents prepared in the lab**.

1. Stains: Record the dye content and adjust the concentration as warranted with new batches in dye.

2. Hematoxylin note: Hematoxylin is light sensitive and subject to oxidation. Stains should be stored in brown or opaque bottles. Oxidation may be slowed by keeping the stain in dishes, covered as much as possible, during staining and when not in use.

D. **Have quality control test for all reagents prepared in the lab.** It is an individual lab's decision on which tests, if any, to perform on commercially purchased reagents.

1. Alcohols (anhydrous and non-anhydrous)
   a)Odor
   b) Color
   c)Specific gravity. This will vary with the formulation of alcohol used and the temperature.

2. Stains
   a)pH b)Color
   (Alum hematoxylin filter paper test): When dropped on a filter paper, it will produce a pattern by diffusion of a maroon color ending in a dark purple edge.
   c)Tissue test

E. Labeling requirements

1. For laboratory-prepared reagents
   a)Name of reagent
   b)Date bottle with preparation date
   c)Initials of the person preparing solution
   d)State expiration date
   e)If applicable, date bottle upon first use.

2. For commercially purchased reagents
   a)Name of reagent
   b)Highlight expiration date
   c)Date bottle received
   d)Date bottle opened/first used

F. In order to have consistently stained sections day after day, it is necessary to make microscopic checks after critical steps in the staining procedure (e.g. after bluing in a progressive program and after the hematoxylin differentiator in a regressive program). **The presence of a functioning microscope is strongly advocated in the staining laboratory.**

Chapter 4

The basics of some common required special stains

Objectives

1- Identify different methods used to detect carbohydrates and mucopolysaccharides (PAS, Microwave alcian blue, microwave alcian blue with hyaluronidase digestion and Modified Putchler Congo Red Amyloid Method with/without sodium permanganate oxidation)
2- Describe different methods to detect connective tissue (Masson's Trichrome stain and Verhoeff's elastic stain)
3- Identify different methods to detect cytoplasmic and nuclear elements (Microwave orcein method for hepatitis b surface antigen and Toluidine blue method for mast cells)
4- Describe different methods to detect pigments and metals (Microwave Rhodanine copper method and Perl's method for ferric iron).
5- Identify different methods to detect nerve cells and fibers (Thioflavin S method for amyloid in neurofibrillary plaques)

Special stains (histochemical methods) are used to help visualize and/or identify structures and substances in sections.

Types of some required special stains in histopathology labs:

1- Different methods used to detect carbohydrates and mucopolysaccharides
-Periodic acid Schiff (PAS).
- Microwave alcian blue.
- Microwave alcian blue with hyaluronidase digestion.
-Modified Putchler Congo Red Amyloid Method with/without sodium permanganate oxidation.
2- Different methods to detect connective tissue.
-Masson's Trichrome stain.
-Verhoeff's elastic stain.
3- Different methods to detect cytoplasmic and nuclear elements.
-Microwave orcein method for hepatitis b surface antigen
-Toluidine blue method for mast cells.
4- Different methods to detect pigments and metals.
-Microwave Rhodanine copper method.
-Perl's method for ferric iron.
5- Different methods to detect nerve cells and fibers.
-Thioflavin S method for amyloid in neurofibrillary plaques

METHODS TO DETECT CARBOHYDRATES AND MUCOPOLYSACCHARIDES:

PAS (PERIODIC ACID SCIFF)

FIXATION: 10% buffered neutral formalin, Zenkers or any good fixative.

TECHNIQUE: Paraffin sections cut at 4 µm.

SOLUTIONS:

1- 0.5% Periodic Acid Solution

Periodic acid ---------- 0.5 gm
Distilled water ---------- 100.0 ml

2- Modified Lillies Schiff Solution

Dissolve 0.3 gm of pararosanilin, in 20 ml of 20% ethyl alcohol. Add 80 ml of distilled water, 2.0 gm of sodium metabisulfite, 0.15 gm of sodium hydrosulfite, and thoroughly mix. Then add 1.0 ml of hydrochloric acid and 0.3 gm of activated charcoal. Stir for 3 minutes and filter through Whatman #1 filter paper. The solution should be water-white. Store Schiffs reagent in a refrigerator at 3-6° C. When stored in a refrigerator, the solution has a shelf-life of at least one year.

3- 0.3% Sodium Borate

Sodium borate (Na2 B4 O7 10H2O) ---------- 0.3 gm
Distilled water ---------- 100.0

METHOD:

1- Deparaffinize and hydrate to distilled water.
2- Oxidize in periodic acid solution for 10 minutes. For kidney, skin and diastase digested liver biopsies, 15 minutes.
3- Rinse in four changes of distilled water.
4- Place in modified Lillies Schiff solution for 15 minutes. For kidney and skin biopsies, 30 minutes.
5- Rinse in four changes of distilled water.
6- Place in 0.3% sodium borate for 15 seconds.
7- Rinse in four changes of distilled water.
8- Stain in acidified Lillie-Mayer hematoxylin for 45 seconds.
9- Rinse in three changes of distilled water.
10- Blue hematoxylin in 0.3% sodium borate for 15 seconds.
11- Rinse in four changes of distilled water.
12- Dehydrate through graded alcohols.
13- Clear in three or four changes of xylene.
14- Mount with synthetic resin.

STAINING RESULTS:

Glycogen, mucin, reticulum, colloid droplets, hyaline deposits in glomeruli, most basement membranes, colloid of pituitary stalks and thyroid give a rose to purple reaction; some types of fungi - red, nuclei - blue.

COMMENT:

Treatment of tissue sections with 0.5% periodic acid results in the formation of aldehyde groupings by cleavage of 1,2 glycols found in various carbohydrates. The presence of the newly formed aldehyde groups is detected by Schiff's reagent (or leucofuchsin). The first stage reaction involves the formation of a colorless, unstable dialdehyde addition compound that is transformed to the colored final product by restoration of the quinoid chromophoric grouping of pararosanilin or other basic fuchsin. This color restoration is usually accomplished by washing in running tap water for 10 minutes after treatment with Schiff's reagent. It is found that treatment with 0.3% sodium borate for 15 seconds accomplishes the same purpose.

The formula for Lillies Schiff reagent has been modified. This modified solution requires less basic fuchsin (pararosanilin) to prepare, can be prepared much faster, yields equally good staining results, and appears to have greater stability than Lillies formula.

Schiff's reagent after decolorization with activated charcoal may be either water clear or have a pale straw color. Some believe that the quality of the activated charcoal determines the final color of the Schiff's reagent. Fresh, activated charcoal is supposed to result in a water clear Schiff's reagent. Other's experience found this is not necessarily true as they have found that Schiff's reagent prepared with different samples of basic fuchsin will not all decolorize completely even when fresh activated charcoal is used. The final color of the Schiff's reagent does not appear to affect the quality or intensity of the Schiff's reaction.

Schiff's reagent can be tested by pouring a few drops of the reagent into 10 ml of reagent grade formaldehyde (37-40%) in a small beaker. If the solution turns reddish purple rapidly, it is good. If the reaction is delayed and the resulting color deep blue-purple, the solution is breaking down and should be discarded.

MICROWAVE ALCIAN BLUE

FIXATION: 10% buffered neutral formalin.

TECHNIQUE: Paraffin sections cut at 5 µm.

SOLUTIONS:

1- 3% Acetic Acid Solution

Acetic acid, glacial ---------- 3.0 ml
Distilled water ---------- 97.0 ml

2- 1.0% Alcian Blue Solution

Alcian blue 8GX, C.I. 74240 ---------- 1.0 ml

3% acetic acid ---------- 100.0 ml

Filter and add a few crystals of thymol.

3- Nuclear Fast Red Solution

Dissolve 0.1 gm nuclear fast red in 100 ml of 5% solution of aluminum sulfate with aid of heat. Cool, filter and add a few grains of thymol as a preservative.

PROCEDURE:

1. Deparaffinize and hydrate to distilled water.
2. Place in 3% acetic acid for 3 minutes.

3. Place in 40 ml of 1.0% alcian blue solution in a glass Coplin jar and microwave at power level 1 (60W) for 3 minutes. Dip the slides up and down several times and allow them to remain in the hot solution (65° C) for 5 minutes.

4. Wash in running tap water for 1 minute and rinse in distilled water.
5. Nuclear fast red solution for 3 minutes.
6. Rinse in three changes of distilled water.
7. Dehydrate in graded alcohols.
8. Clear in three or four changes of xylene.
9. Mount with synthetic resin.

STAINING RESULTS:

Acidic sulfated mucosubstances ---------- blue

Cryptococcus ---------- blue

Nuclei---------- red

COMMENT:

No single staining solution of alcian blue colors all polyanions maximally, but the above procedure, done at pH 2.5, stains all except the most strongly sulfated mucopolysaccharides, and is very useful for demonstrating cells containing mucins in the respiratory tract and in small and large bowel.

Alcian blue probably find sits greatest usefulness in combination stains such as Alcian blue, PAS, and the Kreyberg method.

When heating the alcian blue solution with microwave irradiation the top portion of the solution is warmer by 10-15° C than that near the bottom of the Coplin jar. Therefore, in order to equalize the temperature of the solution the slides are dipped up and down. This assures uniformity of staining throughout the tissue sections.

The stain may be performed at room temperature by staining in the alcian blue solution for 30 minutes.

MICROWAVE ALCIAN BLUE WITH HYALURNIDASE DIGESTION

MATERIAL AND SOLUTIONS

FIXATION: 10% buffered neutral formalin.

TECHNIQUE: Paraffin sections cut at 5 µm.

SOLUTIONS:

1- Phosphate Buffer. pH 6.0

Sodium phosphate, dibasic	0.128	gm
Potassium phosphate, monobasic	0.872	gm
Distilled water	100.0	ml

2- Hyaluronidase Digestion Solution

Hyaluronidase, testicular (Sigma)	0.02	gm
Phosphate buffer, pH 6.0	40.00	ml

Mix just before use.

3- 3% Acetic Acid Solution

(see Microwave Alcian Blue Method)

4- 1.0% Alcian Blue Solution

(see Microwave Alcian Blue Method)

5- 0.1% Nuclear Fast Red Solution

PROCEDURE:

- Use control slide (duplicate section).

1. Deparaffinize and hydrate duplicate sections to distilled water.

2. Place the control and requested section in 40 ml of hyaluronidase digestion solution in a glass Coplin jar and microwave at power level 1 (60W) for 1 minute. Transfer to a 37° C oven for 1 hour. Place the duplicate control and requested slides in 40 ml of phosphate buffer, pH 6.0 in a glass Coplin jar and microwave at power level 1 for 1 minute. Transfer to a 37o C oven for 1 hour.

3. Rinse all the slides in four changes of distilled water.
4. Place in 3% acetic acid solution for 3 minutes.

5. Place in 40 ml of 1.0% alcian blue solution in a glass Coplin jar and microwave at power level 1 (60W) for 3 minutes. Dip the slides up and down several times and allow them to remain in the hot solution (65° C) for 5 minutes.

6. Rinse in three changes of distilled water.
7. Nuclear fast red solution for 3 minutes.
8. Rinse in three changes of distilled water.
9. Dehydrate in graded alcohols.
10. Clear in three or four changes of xylene.
11. Mount with synthetic resin.

COMMENT:

When heating the alcian blue solution with microwave irradiation the top portion of the solution is warmer by 10-15° than that near the bottom of the Coplin jar. Therefore, in order to equalize the temperature of the solution, the slides are dipped up and down. This assures uniformity of staining throughout the tissue sections.

The stain may be performed at room temperature by staining in the alcian blue solution for 30 minutes.

MODIFIED PUCHTLER CONGO RED AMYLOID METHOD WITH/WITHOUT POTASSIUM PERMANGANATE OXIDATION

FIXATION: 10% buffered neutral formalin

TECHNIQUE: Paraffin sections cut at 6 microns.

SOLUTIONS:

1- Modified Weigert's Iron Hematoxylin

Solution A

Hematoxylin, C.I. 75290 ---------- 2.0 gm

Alcohol, 90% ---------- 100.0 ml

Solution B

Ferric chloride, FeCl3 6H2O, 62% aqueous ---------- 4.0 ml

Distilled water ---------- 95.0 ml

Hydrochloric acid, concentrated ---------- 1.0 ml

For use mix equal parts of Solution A and Solution B.

2- 0.5% Acid Alcohol Solution

Sodium Chloride-Alcohol Solution

Sodium chloride ---------- 2.5 gm

Distilled water ---------- 50.0 ml

Absolute alcohol ---------- 50.0 ml

Dissolve the sodium chloride in the distilled water and then add the absolute alcohol. This solution is stable for several months.

3- 1% Potassium Hydroxide

Potassium hydroxide ---------- 1.0 gm

Distilled water ---------- 100.0 ml

4- Congo Red Solution

Sodium chloride-alcohol solution ---------- 50.0 ml

Congo red, C.I. 22120 ---------- 0.1 gm

1% Potassium hydroxide ---------- 0.5 ml

Dissolve the Congo red in the sodium chloride-alcohol solution, then add the hydroxide. Filter through Whatman #4 filter paper. This solution is stable for about one month.

PROCEDURE:

Stain duplicate slides. If only one Congo red is requested, do just one slide and omit steps 2-4.

1. Deparaffinize and hydrate all the slides to distilled water.
2. Oxidize one of each duplicate slide (one from each specimen, one from control block) for 5 minutes in acidified potassium permanganate solution. Hold other duplicate slides on water.
3. Rinse in two changes of distilled water.
4. Decolorize oxidized slides in 1% oxalic acid solution for 5 minutes.
5. Wash oxidized slides in running tap water for 1 minute and rinse in two changes of distilled water.
6. Stain all the slides with modified Weigert's iron hematoxylin for 10 seconds.
7. Wash briefly in running tap water and rinse in two changes of distilled water.
8. Place in acid alcohol solution for 5 seconds.
9. Wash well in running tap water and rinse in two changes of distilled water.
10. Place in 95% alcohol for 5 seconds.
11. Place in Congo red solution for 20 minutes.
12. Dehydrate in three changes of 95% alcohol and four changes of absolute alcohol.
13. Clear in xylene, three or four changes.
14. Mount with synthetic resin.

STAINING RESULTS:
In unoxidized sections, amyloid stains pink to red and demonstrates apple-green birefringence with polarizing microscopy and fluoresces orange to red when viewed with fluorescence microscopy. In oxidized sections, secondary (AA) amyloid shows diminished staining and fluorescence and does not exhibit green birefringence. Primary (AL) amyloid will retain the stain.

COMMENT: Amyloidosis is a group of diseases involving the deposition of insoluble polymerized protein filaments (amyloid) in the interstitial spaces of blood vessels and in various organs including heart, kidneys, lung and liver. Two major types of amyloid exist: primary (AL) and secondary (AA) amyloid. Primary amyloid is characterized by deposition of enzymatically altered immunoglobulin kappa or lambda light chains. Secondary amyloid comprises an enzymatically altered serum protein, which is made in the liver. Deposition of AA amyloid is usually seen in response to chronic inflammatory processes including pulmonary infection, tuberculosis, rheumatoid arthritis, osteomyelitis, leprosy and an occasional neoplasia.

Amyloid can be stained with several dyes including thioflavin T, thioflavin S, crystal violet, methyl violet, sirius red, and the dye of choice, Congo red. Congo red stains amyloid-oriented filaments by deposition of the linear dye molecules with their long axis parallel to protein filaments. This subsequently shows an apple-green birefringence when viewed by polarizing microscopy. We have found that the optimal way of viewing Congo red stained amyloid is by fluorescence microscopy. Small or weakly stained deposits of amyloid, which are often difficult to see by light or polarization microscopy, are readily apparent by fluorescence microscopy. We have, furthermore, not seen significant orange-red fluorescence from any tissue components other than amyloid, except for elastic fibers and coarse collagen fibers.

***STAINS FOR CONNECTIVE TISSUE*:**

<u>MASSONS TRICHROME STAIN</u>

FIXATION: 10% buffered neutral formalin or Bouin's.

TECHNIQUE: Paraffin sections cut at 5 µm.

SOLUTIONS:

1- Bouin's Solution

Picric acid, saturated aqueous ---------- 75.0 ml

Formalin, concentrated, 37-40% ---------- 25.0 ml

Acetic acid, glacial ---------- 5.0 ml

2- Modified Weigert's Iron Hematoxylin

Solution A

Hematoxylin crystals, C.I. 75290 ---------- 2.0 gm

Alcohol, 90% ---------- 100.0 ml

Solution B

Ferric chloride, FeCl3, 6H20, 62% aqueous ---------- 4.0 ml

Distilled water ---------- 95.0 ml

Hydrochloric acid, concentrated ---------- 1.0 ml

3- Working Modified Weigert's Iron Hematoxylin

Equal parts of Solution A and Solution B

4- Biebrich Scarlet-Acid Fuchsin Solution

Biebrich scarlet, C.I. 26905 ---------- 0.45 gm

Acid fuchsin, C.I. 42685 ---------- 0.05 gm

Acetic acid, glacial ---------- 0.50 gm

Distilled water ---------- 50.0 ml

5- Phosphomolybdic-Phosphotungstic Acid Solution

Phosphomolybdic acid ---------- 2.5 gm

Phosphotungstic acid ---------- 2.5 gm

Distilled water ---------- 100.0

6- Aniline Blue Solution

Aniline blue, C.I. 42755 ---------- 1.0 gm

Acetic acid, glacial ---------- 0.8 ml

Distilled water ---------- 40.0 ml

7- 1% Acetic Acid Solution

Acetic acid, glacial ---------- 1.0 ml

Distilled water ---------- 100.0 ml

PROCEDURE:

1. Deparaffinize and hydrate to distilled water.
2. Mordant in Bouin's solution which has been preheated to 58o C for 15 minutes.
3. Wash in running water until the yellow color disappears and rinse in two changes of distilled water.
4. Modified Weigert's iron hematoxylin for 5 minutes.
5. Wash briefly in running water and rinse in two changes of distilled water.
6. Place in 0.5% hydrochloric acid in 70% alcohol for 5 seconds.
7. Wash in running tap water for 30 seconds and rinse in two changes of distilled water.

8. Biebrich scarlet-acid fuchsin solution for 5 minutes.
9. Rinse in three changes of distilled water.
10. Phosphomolybdic-phosphotungstic acid solution for 5 minutes.
11. Aniline blue solution for 20 minutes.
12. Rinse in distilled water.
13. Acetic acid solution for 10 seconds.
14. Rinse in two changes of distilled water.
15. Dehydrate in graded alcohols.
16. Clear in three or four changes of xylene.
17. Mount with synthetic resin.

STAINING RESULTS:

Nuclei ---------- black

Cytoplasm, keratin, muscle fibers and fibrin ---------- red

Collagen ---------- blue

COMMENT:

Treatment of the tissue sections with hot Bouin's solution is essential in order to obtain satisfactory results. Some authors recommend treating the tissue section for 30-60 minutes in hot Bouin's. This sometimes causes tissue sections to become detached from the slides. The 15-minute exposure to hot Bouin's is adequate with much less tendency for tissue sections to detach from the slides.

This stain is mainly ordered for kidney biopsies. The Gomori-trichrome method is used for other tissues because it is easier to perform and provides equally satisfactory staining of collagen and muscle.

VERHOEFFS ELASTIC STAIN

FIXATION: 10% buffered neutral formalin.

TECHNIQUE: Paraffin sections cut at 5 µm.

SOLUTIONS:

1- 5% Alcoholic Hematoxylin

Hematoxylin crystals, C.I. 75290 ---------- 5.0 gm

Alcohol, 80% ---------- 100.0 ml

2- 10% Ferric Chloride

Ferric chloride ($FeCl_3 \cdot 6H_2O$) ---------- 10.0 gm

Distilled water ---------- 100.0 ml

3- Lugol's Iodine Solution

Potassium iodide ---------- 4.0 gm

Iodine ---------- 2.0 gm

Distilled water ---------- 100.0 ml

Dissolve the potassium iodide in a little of the distilled water, add the iodine and dissolve. Add the remainder of the distilled water.

4- Verhoeff's Staining Solution

5% alcohol hematoxylin ---------- 20.0 ml

10% ferric chloride ---------- 10.0 ml

Lugol's iodine ---------- 10.0 ml

Prepare fresh each time.

5- 2% Ferric Chloride

10% Ferric Chloride ($FeCl_3 \cdot 6H_2O$) ---------- 10.0 ml

Distilled water ---------- 40.0 ml

6- Van Gieson's Stain

Acid Fuchsin, C.I. 42685 ---------- 0.1 gm

Picric acid, saturated aqueous (approximately 1.4%) ---------- 100.0 ml

PROCEDURE:

1. Deparaffinize and hydrate to distilled water.
2. Place in Verhoeff's staining solution for 10 minutes.
3. Wash briefly in running tap water and rinse in two changes of distilled water.
4. Differentiated in 2% ferric chloride for 15 seconds. Stop the differentiation with two changes of distilled water. Check microscopically for black elastic fiber staining and gray background. Repeat 2% ferric chloride treatment and distilled water rinses as necessary for adequate elastic fiber demonstration. It is better to slightly under differentiate the tissue, because the subsequent Van Gieson counterstain will extract some of the elastic stain. Brain and heart tissue should be less differentiated with ferric chloride.
5. Rinse in two changes of distilled water.
6. Counterstain in Van Gieson's solution for 1 minute.
7. Dehydrate in three changes of 95% alcohol (in Coplin jars) and complete dehydration in four changes of absolute alcohol.
8. Clear in three or four changes of xylene.
9. Mount with synthetic resin.

STAINING RESULTS:

Elastic fibers ---------- black

Nuclei ---------- blue to black

Collagen ---------- red

Other tissue elements ---------- yellow

COMMENT:

This is the most popular stain for elastic tissue. The only problem is that not every technologist will differentiate the slides the same way in Step #4. Therefore, there will be some variation in the quality of the staining results.

STAINS FOR PIGMENTS AND METAL

PERLS METHOD FOR FERRIC IRON

FIXATION: 10% buffered neutral formalin.

TECHNIQUE: Paraffin sections cut at 5 µm.

SOLUTIONS:

1- 2% Hydrochloric Acid Solution

Hydrochloric acid, concentrated ---------- 2.0 ml

Distilled water ---------- 98.0 ml

2- 10% Triton X-100

Triton X-100 ---------- 10.0 ml

Distilled water ---------- 90.0 ml

Add a few grains of thymol to prevent the growth of fungi.

3- 1% Potassium Ferrocyanide Solution

Potassium ferrocyanide ---------- 1.0 gm

Distilled water ---------- 98.0 ml

10% Triton X-100 ---------- 2.0 ml

4- Hydrochloric Acid-Potassium Ferrocyanide Solution

2% hydrochloric acid ---------- 20.0 ml

1% potassium ferrocyanide ---------- 20.0 ml

Prepare just before use and discard after use.

5- Nuclear Fast Red (Kernechtrot) Solution

Dissolve 0.1 gm nuclear fast red in 100 ml of 5% solution of aluminum sulfate with the aid of heat. Cool, filter, add a few grains of thymol as a preservative.

PROCEDURE:

- Use positive control slides.

1. Deparaffinize and hydrate to distilled water.
2. Place the slides in the hydrochloric acid-potassium ferrocyanide solution for 30 minutes at room temperature.
3. Rinse with five changes of distilled water.
4. Nuclear fast red solution for 3 minutes.
5. Rinse with three changes of distilled water.
6. Dehydrate in graded alcohols.
7. Clear in xylene, three or four changes.
8. Mount with synthetic resin.

STAINING RESULTS:

Ferric iron pigment ---------- bright blue

Nuclei ---------- red

Cytoplasm ---------- pink

COMMENT:

Hemosiderin is a breakdown product of hemoglobin and is thought to be composed of ferric iron and protein. It may be present in tissues in certain pathologic conditions such as hemochromatosis. This yellow-brown pigment is insoluble in alkalis and water but is soluble in acid even after fixation. Treating a tissue section with 10% sulfuric acid overnight will usually remove this pigment.

The principle of Pearl's Prussian blue reaction is that potassium ferrocyanide will form ferric ferrocyanide (Prussian blue) with reactive ferric salts in an acid solution. Dilute hydrochloric acid liberates loosely bound ferric iron from protein.

For many years Gomori's method is used for demonstrating ferric iron. The potassium ferrocyanide and hydrochloric acid solutions used in this method are much stronger that those used in Pearl's method. But Pearl's method is more routinely used because it is more cost effective.

Triton X-100 is added to the potassium ferrocyanide solution because it helps to stabilize the hydrochloric acid-potassium ferrocyanide solution. If Triton X-100 is omitted there is a greater tendency for the solution to break down resulting in a fine blue precipitate forming in the Coplin jar and sometimes on the slides and tissue sections.

MICROWAVE RHODANINE COPPER

FIXATION: 10% buffered neutral formalin.

TECHNIQUE: Paraffin sections cut at 5 µm.

SOLUTIONS:

1- 0.4% Rhodanine Solution (prepare fresh)

5 – (p-dimethylaminobenzylidene) rhodanine ---------- 0.024 gm

Absolute alcohol ---------- 6.000 ml

2- 1% Sodium Acetate Solution

*Sodium acetate trihydrate ---------- 1.0 gm

Distilled water ---------- 100.0 ml

*If anhydrous sodium acetate is used, use 0.6 gm.

Rhodanine Solution (Working)

6- Rhodanine solution (stock) ---------- 5.0 ml

1% sodium acetate ---------- 45.0 ml

Filter the rhodanine solution through Whatman #4 filter paper (70 mm in diameter) into the 1% sodium acetate.

7- 0.4% Sodium Borate

Sodium borate, ($Na_2B_4O_7 \cdot 10 H_2O$) ---------- ----- 0.4 gm

Distilled water ---------- 100.0 ml

PROCEDURE:

- Use positive control slide.

1. Deparaffinize and hydrate to distilled water.
2. Place slides in the working rhodanine solution in a plastic Coplin jar, with the cap loosely applied, and microwave at power level 6 (360W) for 30 seconds. Agitate for about 15 seconds. Return the Coplin jar to the microwave oven and microwave at power level 6 for 35 seconds. Agitate for about 15 seconds and allow the slides to remain in this hot solution (about 80o C) for 5 minutes.
3. Return the Coplin jar to the microwave oven and microwave at power level 6 for 10 seconds. Agitate for about 15 seconds and allow the slides to remain in the hot solution for 5 minutes.
4. Wash with six changes of distilled water.
5. Place in acidified Lillie-Mayer hematoxylin for 10 seconds.
6. Rinse with two changes of distilled water.
7. Blue hematoxylin in 0.4% sodium borate for 15 seconds.

8. Rinse with four changes of distilled water.
9. Mount with Apathy's aqueous mounting media.

STAINING RESULTS:

Copper ---------- bright red or rust-red intracytoplasmic granules

Nuclei ---------- blue

COMMENT:

This stain is found to be both sensitive and specific and to allow for a semiquantitative evaluation of the amount of copper present (see Irons, et al). This microwave method may not always give as good results as the overnight method which is done in a 37 C oven. A good positive control of this method is any fetal liver of the third trimester fixed in buffered neutral formalin for not longer than 24 hours prior to paraffin processing. Fetal liver of the third trimester contains copper within hepatocytes. Care must be taken not to over counterstain with hematoxylin which will mask the positive staining of the copper.

METHODS FOR CYTOPLASMIC AND NUCLEAR ELEMENTS:

MICROWAVE ORCEIN METHOD FOR HEPATITIS B SURFACE ANTIGEN

FIXATION: 10% buffered neutral formalin.

TECHNIQUE: Paraffin sections cut at 5 µm.

SOLUTIONS:

1- 0.3% Acidified Potassium Permanganate (Stock)

Potassium permanganate ---------- 0.3 gm

Distilled water ---------- 100.0 ml

Sulfuric acid, concentrated ---------- 0.2 ml

For use dilute with equal parts of distilled water.

2- 1% Oxalic Acid Solution

Oxalic acid ---------- 1.0 gm

Distilled water ---------- 100.0 ml

3- 1% Orcein Solution

Orcein, C.I. (Ed. 1) 1242 ---------- 1.0 gm

Alcohol, 70% ---------- 100.0 ml

Hydrochloric acid, concentrated ---------- 1.0 ml

Let solution stand at room temperature for at least one week before use

PROCEDURE:

- Use control slide.

1. Deparaffinize and hydrate to distilled water.
2. Place in diluted acidified potassium permanganate for 5 minutes.
3. Rinse in two changes of distilled water.
4. Place in 1% oxalic acid for 2 minutes.
5. Wash in running tap water for 1 minutes.
6. Rinse in two changes of distilled water.
7. Dip several times in 70% alcohol.
8. Place in 45 ml of orcein solution in a glass Coplin jar and microwave at power level for 1 ½ minutes. Transfer to a 37o oven for 2 hours.
9. Rinse slides in four changes of distilled water.
10. Dehydrate in graded alcohols.
11. Clear in xylene, three or four changes.
12. Mount with synthetic resin.

STAINING RESULTS:

Hepatitis B surface antigen (HbsAg) ---------- brown (diffuse cytoplasmic)

Copper binding protein ---------- brown, fine cytoplasmic granules

Elastic fibers ---------- brown

COMMENT:

Some orcein dyes do not give satisfactory staining results. Dyes which are not satisfactory will either overstain the section or not adequately stain the HbsAg. Dyes certified as orcein by the Biological Stain Commission are tested for their ability to stain HbsAg. The stain may be done at room temperature by staining for 4-8 hours in the orcein solution.

<u>TOLUIDINE BLUE FOR MAST CELLS</u>

FIXATION: 10% buffered neutral formalin.

TECHNIQUE: Paraffin sections cut at 5 µm.

SOLUTIONS:

1- 0.5% Potassium Permanganate Solution

Potassium permanganate ---------- 0.5 gm

Distilled water ---------- 100.0 ml

2- 1% Potassium Metabisulfite Solution

Potassium metabisulfite ---------- 1.0 gm

Distilled water ---------- 100.0 ml

3- 0.02% Toluidine Blue Solution

Toluidine Blue 0, C.I. 52040 ---------- 0.02 gm

Distilled water ---------- 100.0 ml

Acetic acid, glacial ---------- 0.25 ml

PROCEDURE:

- Use control slide.

1. Deparaffinize and hydrate to distilled water.
2. Potassium permanganate solution for 2 minutes.
3. Rinse in two changes of distilled water.
4. Potassium metabisulfite solution for 1 minute.
5. Wash in running tap water for 3 minutes.
6. Rinse in two changes of distilled water.
7. Toluidine Blue solution for 5 minutes.
8. Rinse in three changes of distilled water.
9. Dehydrate in graded alcohols.
10. Clear in xylene, three or four changes.
11. Mount with synthetic resin.

STAINING RESULTS:

Mast cell granules ---------- purple (metachromatic)

Acid mucopolysaccharides ---------- red to pink

Nuclei ---------- blue

COMMENT:

This staining method appears to minimize background staining, which makes it much easier to locate and identify mast cells.

METHODS FOR NERVE CELLS AND FIBRES:

<u>THIOFLAVIN S METHOD FOR AMYLOID IN NEUROFIBRILLARY PLAQUES</u>

FIXATION: 10% buffered neutral formalin.

TECHNIQUE: Paraffin sections cut at 6-8 µm.

SOLUTIONS:

1- 1% Thioflavin S

Thioflavin S, C.I. 49010 ---------- - 1.0 gm

Distilled water ---------- 100.0 ml

2- 1% Thioflavin T

Thioflavin T, C.D. 49005 ---------- 1.0 gm

Distilled water ---------- 100.0 ml

3- Modified Weigert's Iron Hematoxylin

(See Modified Puchtler's Congo Red Method)

4- 0.5% Acid Alcohol Solution

(See Modified Puchter's Congo Red Method)

5- 1% Acetic Acid

Acetic acid ---------- 1.0 ml

Distilled water ---------- 100.0 ml

PROCEDURE:

1. Deparaffinize and hydrate to distilled water.
2. Place in modified Weigert's iron hematoxylin for 15 seconds.
3. Rinse in three changes of distilled water.
4. Place in 0.5% acid alcohol for 5 seconds.
5. Rinse in four changes of distilled water.
6. Stain with 1% thioflavin S for 5 minutes. For all other tissues stain in 1% thioflavin T for 5 minutes.
7. Wash well in running water.
8. Place in 1% acetic acid for 15 minutes.
9. Rinse in three changes of distilled water.
10. Stand slide on end and thoroughly air dry.
11. Dip in xylene and mount with synthetic resin.

STAINING RESULTS:

When thioflavin S is viewed with a fluorescence microscope amyloid in neurofibrillary plaques and in blood vessels will fluoresce intense yellow-white. Amyloid in tissues stained with thioflavin T will fluoresce an intense white.

COMMENT:

Thioflavin S stains amyloid in neurofibrillary plaques in brain much better than thioflavin T. However, there is considerable non-specific background fluorescence. Thioflavin T stains amyloid well in other tissues and there is very little background fluorescence.

Most texts that contain the thioflavin S and thioflavin T methods use alum hematoxylin to quench background fluorescence. It is found that Weigert's iron hematoxylin is more effective in quenching background fluorescence.

Chapter 5
The basics of Immunohistochemistry

Objectives

1. Describe the different steps of staining techniques.
2. Enumerate the possible staining artifacts.
3. Identify the solutions for those artifacts

History

The principles of IHC have been known since the 1930s, but it was not until 1942 that the first IHC study was reported. Since then, major improvements have been made in tissue fixation and sectioning methods, antigen/epitope retrieval, antibody conjugation, immunostaining methods and reagents, as well as microscopy itself. As a result, IHC has become a routine, but essential tool in diagnostic and research laboratories (figure 14).

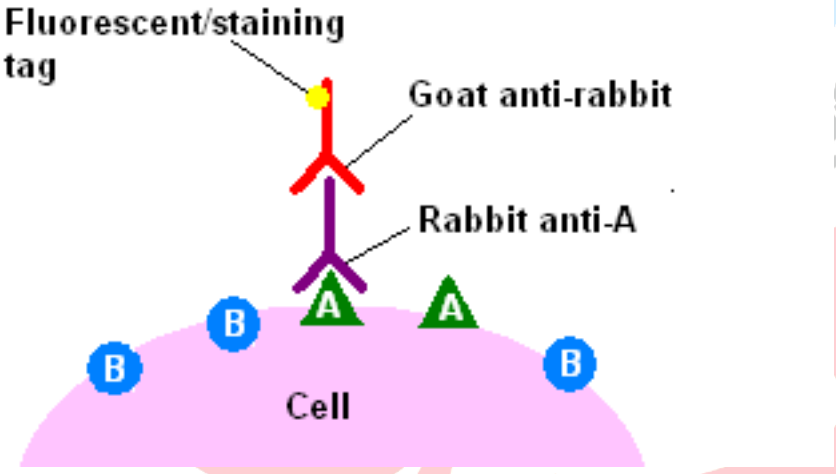


Figure (14): Schematic illustration for the mechanism of immunohistochemistry

Applications

IHC is used for:

1- Disease diagnosis, for example, using specific tumor markers, physicians use IHC to diagnose if a tumor is benign or malignant, to determine its stage and grade, and to identify the cell type and origin of a metastasis in order to find the site of the primary tumor. A variety of other non-neoplastic diseases and conditions are diagnosed using IHC as a primary tool or as a confirmatory procedure.
2- Biological research; IHC can be used alone or in conjunction with other analytical techniques to study, for example, normal tissue and organ development, pathological processes, wound healing, cell death and repair, and many other fields.
3- Drug development. IHC is also used in drug development to test drug efficacy by detecting either the activity or the up- or down-regulation of disease markers in the target tissues and elsewhere.

Traditional IHC is based on the immunostaining of thin sections of tissues attached to individual glass slides. Multiple small sections can be arranged on a single slide for comparative analysis, a format referred to as **a tissue microarray.**

Typically, IHC slides are prepared, processed, and stained manually or in small groups. However, current technology provides automation (figure 15) options for high-throughput sample preparation and staining. Samples can be viewed by either light or fluorescence microscopy, and advances in the last 15 years have improved the ability to capture images, quantitate the data, and increase the collection of that data through high content screening.

Figure (15): Automated immune-stainer.

STEPS:

Tissue fixation

Most tissue fixatives chemically crosslink proteins and/or reduce protein solubility, which can mask target antigens during prolonged or improper fixation. Therefore, the right fixation method must be optimized based on the application and the target antigen to be stained.

The most common fixative is formaldehyde (formalin). Tissues fixed in formaldehyde are typically embedded in paraffin wax to permit sectioning and further processing (see below). Such tissues and the sections cut from them are often referred to as formalin-fixed and paraffin-embedded or FFPE. Although formaldehyde is the most commonly used fixative, many other fixatives can also be used (e.g. acetone, methanol). Generally, use of these alternative fixatives depends on how the target antigens react to fixation in the first place.

Tissue embedding

Formalin-fixed tissue samples are usually embedded in paraffin to maintain their natural shape and tissue architecture during long-term storage and to facilitate sectioning prior to IHC. Such samples and the sections prepared from them are usually referred to as formalin-fixed, paraffin-embedded (FFPE) materials.

Samples which are too sensitive for either chemical fixation or the solvents used to remove the paraffin, can be encased in a cryogenic embedding material and then snap-frozen in liquid nitrogen. Thin slices of these frozen tissue samples are sectioned on a cryostat (freezing microtome), transferred to slides, and then dried to preserve morphology. Such sections are referred to as frozen or cryosections.

Sectioning and mounting

Paraffin wax is the most commonly used embedding medium for routine histological applications, and formalin-fixed, paraffin-embedded (FFPE) sections produce satisfactory results for detecting most tissue antigens using standard antigen retrieval techniques. However, some antigens are destroyed during routine fixation and paraffin embedding—in which case, frozen tissue sectioning becomes the method of choice.

The disadvantages of frozen sectioning include, but are not restricted to these limitations:

1- Poor morphology.
2- Decreased resolution at high magnifications.
3- Special storage needs.

FFPE tissues are usually cut into sections as thin as 4 to 5 μm with a microtome. These sections are then mounted onto glass slides that are coated with a tissue adhesive. This adhesive is commonly added by surface-treating glass slides with 3-aminopropyltriethoxysilane (APTS) or poly-L-lysine, both of which leave amino groups on the surface of the glass to which the tissue adheres. In the past, and now, if necessary, slides can be coated with actual adhesives, including gelatin, egg albumin or even Elmer's glue. After mounting, the sections are dried in an oven or microwaved in preparation for de-paraffinization.

Frozen sections are cut using a pre-cooled cryostat and mounted to adhesive-coated glass slides. These sections are often dried overnight at room temperature and are usually post-fixed by immersion in pre-cooled (-20°C) acetone, fresh paraformaldehyde, or formaldehyde/formalin at ambient temperature. The drying step is sometimes skipped depending on the target antigens and tissue being used.

The paraffin in FFPE sections must be completely removed before IHC staining. If de-paraffinization is not complete, the target antigens will be obscured and the antibodies will be unable to react with them. In fact, paraffin's hydrophobicity actually repels aqueous solutions containing the IHC staining reagents. Flammable, toxic, and volatile organic solvent xylene has traditionally been used to de-paraffinize FFPE slides, although xylene-free de-waxing alternatives are now available.

Formaldehyde fixation generates methylene bridges that covalently crosslink proteins in tissue samples. These bridges can mask antigen and/or epitope accessibility and inhibit or prevent antibody binding. As a result, FFPE sections typically require treatment designed to unmask or retrieve the antigenic epitopes prior to staining. This is called epitope or **antigen retrieval.**

Antigen Retrieval

1- Epitope/antigen retrieval is usually performed by heating or boiling the de-paraffinized sections in various buffers at different pH values, which is called heat-induced epitope retrieval or HIER.
2- Antigens can also be retrieved by digesting the tissue sections with a proteolytic enzyme like pepsin, trypsin, or proteinase K. If antigen or epitope-specific retrieval conditions are not already documented in the literature or on our antibody data sheet, an effective method must be determined empirically. It is also necessary to mention that although thorough de-paraffinization is always required prior to IHC staining, antigen or epitope retrieval is not. In some FFPE tissues, certain individual antigens are not obscured, so a retrieval step is not required prior to staining.

Quenching/blocking endogenous target activity

Many popular staining approaches depend on biotin and its binding proteins like streptAvidin (SA), NeutrAvidin (NA), and avidin (AV). Also, most detection strategies employ horseradish peroxidase (HRP) or alkaline phosphatase (AP) activity for enzyme-mediated detection of target antigens in the presence of specific substrates. Thus, inactivating (quenching) or masking endogenous forms of these proteins prevents false positive detection and high background staining. The general strategies include physically blocking or chemically inhibiting all endogenous biotin or enzyme activity, respectively

Blocking nonspecific sites

Although antibodies show preferential avidity and affinity for specific epitopes, antibodies may partially or weakly bind nonspecifically to sites on non-antigen proteins that mimic the correct binding sites on the target antigen. In the context of antibody-mediated antigen detection, nonspecific binding causes high background staining that can mask the detection of the target antigen. To reduce background staining in IHC, ICC, and any other immunostaining application, prior to staining, the samples are incubated with a buffer that blocks the non-specific sites to which the primary or secondary antibodies may otherwise bind. Common blocking buffers include some percentage of normal serum, non-fat dry milk, BSA (bovine serum albumin), gelatin, and one or more gentle surfactants to aid in wetting. Many commercial blocking buffers with proprietary formulations are available for greater blocking efficiency.

Immunodetection

Detecting the target antigen with antibodies is a multi-step process that requires optimization at every level to maximize signal detection.

Both primary and secondary antibodies are diluted into a buffer formulated to help stabilize the antibody, promote its uniform and complete diffusion into the sample, and discourage nonspecific binding. While one diluent may work with one antibody, the same diluent may not work with another antibody, demonstrating the need for optimization for each one.

Rinsing the sample in between antibody applications is critical to remove unbound antibodies and also to remove antibodies that are weakly bound to nonspecific sites. Rinse buffers are usually simple solutions with only a few components, but the right components must be considered to maximize washing efficiency and minimize interference with signal detection.

Antibody-mediated antigen detection approaches are separated into direct and indirect methods. Both of these methods use antibodies to detect the target antigen, but the selection of the best method to use depends on the level of target antigen expression, its accessibility, and the type of readout desired. Most indirect methods employ the inherent binding affinity of streptavidin and related proteins for biotin to detect a biotinylated antibody that is bound to the target antigen. The antigen-bound antibody is then localized by adding an enzyme-conjugated streptavidin conjugate which generates an amplified signal when appropriate substrates are added. IHC target antigens are detected directly through either chromogenic or fluorescent means, and the type of readout depends on the experimental design. Chromogenic detection is based on antibodies conjugated to enzymes. Most often, the enzymes used are horseradish peroxidase (HRP) or alkaline phosphatase (AP), which are conjugated to primary or secondary antibodies. When incubated with appropriate substrates, the enzyme activity leads to the precipitation of insoluble, colored precipitates at the antigen localization site. Such chromogenic, precipitating substrates include DAB and AEC for HRP, and Fast Red and NBT/BCIP (rarely used) for AP, respectively. For fluorescence detection, the primary or secondary antibody is conjugated to a fluorophore that is detected by fluorescent microscopy.

Counterstains

It provides contrast to the primary stain and can be cell structure-specific. These single-step stains are usually added after antibody staining. Common counterstains include hematoxylin, eosin, nuclear fast red, methyl green, DAPI, and Hoechst fluorescent stain.

Sealing the stained sample

After all staining is completed, the sample should be preserved for archiving purposes and to prevent enzymatic product solubilization or fluorophore photo-bleaching. Sealing the sample by mounting a coverslip with an appropriate mounting solution (mountant) stabilizes the tissue section and the stain. An anti-fade reagent should also be included if fluorescent detection was used to prolong fluorescence excitation. The coverslip can then be sealed with clear nail polish or a commercial sealant after the mountant has cured to prevent sample damage. Mountants with organic and aqueous formulations are commercially available.

ARTIFACTS AND TROUBLESHOOTING:

Strong background staining

The following points are provided to help identify the cause of high background staining, which results in a poor signal-to-noise ratio.

Cause: Endogenous enzymes

Incubate a test tissue sample with the detection substrate alone for a length of time equal to that of the antibody incubation. A strong background signal suggests interference from endogenous peroxidases or phosphatases.

Solution: Quench endogenous peroxidases with 3% H2O2 in methanol or water or use a commercial kit.

Endogenous phosphatases can be inhibited with the endogenous alkaline phosphatase inhibitor, levamisole.

Cause: Endogenous biotin or lectins

High background can occur when endogenous biotin is not blocked prior to adding the avidin–biotin–enzyme complex.

Solution: Two basic steps are involved in this blocking procedure:

Bind all endogenous biotin moieties with excess streptavidin (or equivalent biotin-binding protein); wash thoroughly.

Block remaining streptavidin biotin-binding sites with free biotin; wash thoroughly.

The second step is necessary because streptavidin, avidin and NeutrAvidin Proteins are tetrameric proteins, having four biotin-binding sites per molecule. Therefore, the first step in which endogenous biotin is blocked by excess streptavidin must be followed by a second step in which excess biotin-binding sites on the bound streptavidin are blocked with free biotin. If this second step were not performed, the blocking streptavidin would undesirably bind the biotin-labeled probe used in the assay. After the final wash, the result is a sample in which all biotin molecules are bound by streptavidin and all biotin-binding sites on streptavidin are bound by biotin.

If the ABC complex is made with avidin, the highly-glycosylated protein can bind to lectins in the tissue sample.

- **Solution:** Block endogenous lectins with 0.2 M alpha-methyl mannoside in dilution buffer. Alternatively, use streptavidin or Thermo Scientific NeutrAvidin Protein instead of avidin, because both are not glycosylated and won't bind to lectins.

Cause: Secondary antibody cross-reactivity or nonspecific binding

The secondary antibody may show a strong or moderate affinity for identical or similar epitopes on non-

target antigens.

Solution: If normal serum from the source species for the secondary antibody is used to block the tissue, then increase the serum concentration to as high as 10% (v/v), if necessary. If you are blocking with another reagent (BSA, nonfat dry milk), then add 2% (v/v) or more normal serum from the source species for the secondary antibody. Alternatively, reduce the concentration of the biotinylated secondary antibody.

Egg white, which contains avidin, was often used to coat slides, dilute antibodies or block tissue samples because it is a readily available and inexpensive source of carrier proteins. It is used very rarely nowadays.

- **Solution:** Avoid using egg whites to prevent egg white–based avidin from binding biotinylated secondary antibody during IHC staining. Synthetic tissue adhesives as well as avidin-free antibody diluents and blocking buffers are readily available.

Cause: Issues with the primary antibody

Nonspecific interactions between the primary antibody and non-target epitopes in the tissue sample occur regularly during incubation but at a level that does not influence background staining. A high primary antibody concentration will increase these interactions and thus increase nonspecific binding and background staining.

- **Solution:** Reduce the final concentration of the primary antibody used for staining.

The primary antibody may also show a strong or moderate affinity for identical or similar epitopes on non-target antigens.

- Solution: Increase the blocking buffer composition and/or concentration, or use a different primary antibody.

The primary antibody diluent may contain little or no NaCl, which helps to reduce ionic interactions.

- Solution: Add NaCl to the blocking buffer/antibody diluent so that the final concentration is between 0.15 M and 0.6 M NaCl. The best NaCl concentration to use will have to be determined empirically.

Additional notes on reducing high background staining

- **Carefully prepare tissue sample.** Damage to the tissue can cause diffuse staining.
- **Prepare thinner sections** if penetration of the detection reagents is insufficient.
- **Optimize fixation**. Each tissue antigen will react differently with different fixatives. Optimize the pH, incubation time and temperature.
- **Blocking may be improved by simply draining the excess buffer** instead of washing the tissue sample prior to the addition of antibodies.
- **Use a monoclonal primary antibody** instead of a polyclonal to reduce cross-reactivity.
- **Use cross-adsorbed polyclonal antibodies** to reduce cross-reactivity.

- **Affinity-purify the antibody preparation on an immobilized antigen column**. Many primary antibodies and almost all secondary antibodies are purified in this manner by their suppliers.
- **Decrease the concentrations of the primary and/or secondary antibodies** to reduce nonspecific binding.
- **Decrease the incubation times** with the primary and secondary antibodies to reduce nonspecific binding.
- **Choose an improved substrate** that will produce a higher signal-to-noise ratio for the system such as metal-enhanced DAB rather than DAB, or kits based on TSA technology.

Weak target staining

Cause: Enzyme–substrate reactivity

Even when the tissue sample is properly prepared and labeled, the enzyme–substrate reaction must occur for the chromogenic precipitate to form. Deionized water can sometimes contain peroxidase inhibitors that can significantly impair enzyme activity. Additionally, buffers containing sodium azide should not be used in the presence of HRP. Finally, the pH of the substrate buffer must be appropriate for that specific substrate.

A simple test to verify that the enzyme and substrate are reacting properly is to place a drop of the enzyme onto a piece of nitrocellulose and then immediately dip it into the prepared substrate. If the enzyme and substrate are reacting properly, a colored spot should form on the nitrocellulose.

- **Solution:** Change the enzyme diluent and/or prepare substrate at the proper pH and repeat the test.

Cause: Primary antibody potency

Primary antibodies generally lose affinity for the target antigen over time, either due to protein degradation or denaturation caused by long-term storage, microbial contamination, changes in pH or harsh treatments (e.g., freeze/thaw cycles).

Test the primary antibody for potency by staining tissue samples known to contain the target antigen with various concentrations of the primary antibody; do the test concurrently with the test sample. If the positive control is not positive for the target antigen at all, then this suggests that the primary antibody has lost potency. In fact, it is good laboratory practice to always run a positive control sample through your staining protocol along with the experimental samples.

- **Solution:** Ensure that the antibody diluent pH is within the specified range for optimum antibody binding (7.0 to 8.2) and that the antibody is stored according to the manufacturer's instructions. To prevent contamination of your antibody solutions, wear gloves when dispensing antibodies, and use sterile pipette tips, if appropriate. Even if you store your antibodies in a refrigerator, always divide them into separate small aliquots. This prevents contamination or loss of the whole vial of antibody if a problem arises.

Cause: Secondary antibody inhibition

While high concentrations of the secondary antibody can increase background staining, extremely high concentrations can have the opposite effect and reduce antigen detection.

To test if the secondary antibody concentration is inhibitory, stain positive control samples using decreasing concentrations of the secondary antibody. An increase in signal as the concentration decreases suggests that antibody concentration is too high.

- **Solution:** Reduce the concentration of the secondary antibody.

If the diluent and/or blocking solution contains antigen-neutralizing antibodies, such as those found in serum, then the antibodies will block secondary antibody binding.

- **Solution:** Remove the neutralizing antibodies or change to a different diluent and/or blocking solution.

Additional notes on increasing staining intensity

- **Optimize fixation**. The immunoreactivity can be affected by the fixative step along with the processing step. Avoid freeze/thaw cycles and high temperatures if the antigen is susceptible.
- **Use clean slides for mounting of tissue,** and use appropriate conditions to prevent tissue from falling off the slides during processing.
- **Do not inhibit enzyme activity**. If an AP system is being used, do not use phosphate buffer. If an HRP system is being used, do not use sodium azide. Both will inhibit the enzyme activity.
- **Do not over-block the tissue**, since antigenic sites may be masked. In fact, excessive blocking can be worse than not enough or even no blocking.
- **Remember that neutralizing antibodies may be in the serum added to the blocking buffer.**
- **Screen potential antibodies using a membrane-based system**, such as dot-blots. If you use an ELISA to evaluate antibodies, bear in mind that protein conformations can be altered by adsorption to the surface of the wells in plastic 96-well plates. This means that it is possible that some monoclonal antibodies selected in an ELISA will not recognize native protein in the tissue.
- **Increase antibody penetration of the tissue** by using unmasking agents such as trypsin, pepsin, chymotrypsin and Pronase. Additionally, try permeabilizing the sections with a buffered solution of Triton X-100 (0.1–1% (v/v) prior to staining.
- **Increase the detection efficiency**, and possibly the sensitivity, by using signal amplification systems.
- **Properly prepare enzyme complex**. Carefully mix all components of the enzyme–substrate complex in the correct proportions. Try to prepare dilutions of the enzyme conjugates right before use and don't re-use them because enzyme activity is labile in dilute solutions. Never freeze diluted enzyme conjugates in an attempt to prolong their shelf life.
- **Avoid potential sources of biotin**, such as nonfat dry milk or Fraction V-grade BSA (use only IHC-grade).
- **Increase incubation times or concentrations of the primary or secondary reagents**.
- **Use a more sensitive substrate** system such as a metal-enhanced DAB substrate.
- **Always run a positive control** to determine if the system is working.
- **Use the correct counterstain and mountant**. Some enzymatic products are soluble in alcohol, xylene or other solvents (e.g., aminoethyl carbazole (AEC)). Consider an aqueous mountant in such cases.

Autofluorescence

If a fluorescent marker is being used, check to make sure that there is no autofluorescence in the unprocessed, fixed tissue. In particular, FFPE sections often show strong autofluorescence that may be difficult to inhibit. Many of the options listed above can then be tested to identify the cause of autofluorescence.

If there is autofluorescence in the test sample, then this suggests that either the tissue sample shows inherent autofluorescence (which is common) or that the fixation method is causing the sample to autofluoresce. To determine if the fixation step is the cause of the autofluorescence, test different fixatives (i.e., if aldehyde fixation is used, try a non-aldehyde fixative) to determine if autofluorescence can be reduced without sacrificing antigen detection. If aldehyde fixation is used and no other fixative can be used, then fixative-induced autofluorescence may be reduced by treating the sample with ice cold sodium borohydride (1 mg/mL) in PBS or TBS.

Another approach to reducing autofluorescence is to treat the tissue sample with dyes that quench fluorescence. These dyes include:

1. Pontamine sky blue
2. Sudan black
3. Trypan blue

Paraffin-embedded samples are often more autofluorescenct, even though the sample has been thoroughly de-waxed. Under the circumstances, switching to frozen sections may reduce autofluorescence.

If these approaches are not sufficient to reduce autofluorescence while maintaining tissue sample detection, then the only other alternative is to just choose a fluorescent marker that will not compete with the autofluorescence. For example, fluorescence from dyes that emit at near-infrared wavelengths, such as Invitrogen Alexa Fluor 647, Alexa Fluor 680, Alexa Fluor 750 and Alexa Fluor 790, are not affected by most tissue autofluorescence.

Chapter 6
Transmission Electron Microscopy

Objectives

1-Identify the technique of sample fixation and preparation.
2-Describe the technique of sectioning and routine staining.
3-Identify the technique of immunogold labeling and cryo-technique.
4-Enumerate the artifacts and how to resolve,

Sample preparation for Transmission electron microscopy (TEM)

TEM is a microscopy technique whereby a beam of electrons is transmitted through an ultrathin specimen, interacting with the specimen as it passes through it. An image is formed from the electrons transmitted through the specimen, magnified and focused by an objective lens and appears on an imaging screen, a fluorescent screen in most TEMs, plus a monitor, or on a layer of imaging plate, or to be detected by a sensor such as a CCD camera.

Biological materials contain large quantities of water. To be able to view it in the electron microscopy, the first stage in preparing is the fixation, one of the most important and most critical stages. We need to fix it in a way that the ultrastructure of the cells or tissues remain as close to the living material as possible. The sample is then dehydrated through an acetone or ethanol series, passed through a "transition solvent" such as propylene oxide and then infiltrated and embedded in a liquid resin such as epoxy and LR White resin. After embedding the resin block is then thin sectioned by a process known as ultramicrotomy (figure 16), sections of 50 - 70 nm thickness are collected on metal mesh 'grids' and stained with electron dense stains before observation in the TEM. Sectioning the sample allows us to look at cross-sections through samples to view internal ultrastructure. Many modifications to the basic protocol can be applied to achieve many different goals, immune-gold labeling for example; the in situ localization of specific tissue constituents using antibody and colloidal gold marker systems.

FIXATION:

Tissue can be fixed by immersion. Fixation time is variable, depending on tissue, but usually from 4 hours to overnight at 4 degrees (refrigerator).

1-Primary Fixative: 1/2 strength Karnovsky's.

-2% Paraformaldehyde/2.5% Glutaraldehyde

-This is buffered with a 0.2M Cacodylate Buffer

Note: Always use the fixative in the fume hood and wear gloves. Glutaraldehyde fixes skin and cacodylate buffer contains arsenic.

2-0.1M Cacodylate Buffer wash

3-Post Fixative: 2% aqueous OsO4/0.2 M Cacodylate Buffer

• A 1:1 solution for 2 hours in the refrigerator

4- 0.1 M Cacodylate Buffer wash

DEHYDRATION:

Dehydration with ethanol (15 minutes and decant):

35% ETOH ,50% ETOH, 70% ETOH, 95% ETOH, 100%ETOH, 100% ETOH Propylene Oxide (two settings), 1:1 PO/Epon resin (overnight)

-The dehydrating times should be adjusted to size and kind of tissue.

-The resin is an Epon 812 recipe

EMBEDDING:

Embedding with Epon:

-Next morning, change out to fresh Epon 812 for 1-3 hours.

-Embed (always) in fresh Epon 812

Polyethylene capsules are placed in a holder and numbered strips of paper are inserted. A drop of fresh Epon 812 is placed in the capsules and the specimen is transferred to the appropriate capsule.

The “blocks” are cured for 48 hours in a 60 degree oven.

Sectioning with ultramicrotome

Materials for TEM must be specially prepared to thicknesses which allow electrons to transmit through the sample, much like light is transmitted through materials in conventional optical microscopy. Because the wavelength of electrons is much smaller than that of light, the optimal resolution attainable for TEM images is many orders of magnitude better than that from a light microscope.

The block is cut into semithin sections (1 μm) with a glass knife, using an ultramicrotome (figure 16). The sections are then stained with Toluidine Blue for LM for orientation, and for selecting of a small area for ultrathin sectioning. Ultrathin sections are made at 50-70 nm using a diamond knife and placed/collected on a grid of metal.

Support film on TEM grids

Formvar film is useful for the support of ultrathin sections on the finer mesh grids. Using of support film

are ideal for those applications requiring large viewing areas without grid bar interference. These films must be strong, clean and remain attached to the specimen grids during specimen preparation.

A Formvar film covered with a "light" layer of carbon will help to stabilize the film when the film is exposed to the electron beam.

Positive staining

Details in light microscope samples can be enhanced by stains that absorb light; similarily TEM samples of biological tissues can utilize high atomic number stains to enhance contrast. The stain absorbs electrons or scatters part of the electron beam which otherwise is projected onto the imaging system. Uses heavy metals such as lead and uranium to scatter imaging electrons and thus give contrast between different structures, since many (especially biological) materials are nearly "transparent" to electrons (weak phase objects).

Heavy metal salts attach to various organelles or macromolecules within the sections to increase their electron density and they appear dark against a lighter background. Uranyl ions react strongly with phosphate and amino groups so that nucleic acids and certain proteins are highly stained. Lead ions bind to negatively charged components and osmium-reacted areas (membranes).

Grids are stained with heavy metals, such as uranyl acetate and lead citrate. The grids, with the specimen side down, remain in 4% uranyl acetate for 25 minutes and are then rinsed in a series of four beakers of pure water. After rinsing, the grids are then stained with 1% lead citrate for 5 minutes, rinsed again in pure water, and stored in a grid box.

** This procedure is the basic TEM procedure. Each and every sample should be evaluated and the procedure is adjusted to make sure the best results will be obtained.

Immunogold labelling

This technique uses antibodies to detect the intracellular location of structures of particular proteins by electron microscopy. Ultrathin sections are labelled with antibodies against the required antigen and then labelled with gold particles. Gold particles of different diameters enable two or more proteins to be studied.

EM Lab can offer post-embedding immunogold labelling of samples in resin (Epoxy, LR White and Lowicryl) and on frozen hydrated ultrathin sections (Tokuyasu-method).

The investigator must supply the primary and secondary antibodies. The investigator should do immunolabelling at the fluorescent light microscopy level before attempting it at the EM level.

Cryo techniques/Low temperature

Cryo-ultramicrotomy is the ultrathin sectioning of unfixed/fixed, cryo-protected and/or rapidly frozen samples at very low temperatures from -15 to -160□C.

Freeze substitution is a process where the water molecules within the samples are exchanged with a solvent (usually methanol or acetone), then, the solvent with a resin (Lowicryl, LR White or Epoxy resins). This method, working at temperatures below 0oC, reduces the loss of components from the sample and minimizes the denaturization of the proteins. In the end, the sample is fully infiltrated with

pure resin. Polymerization of the resin is performed outside (Epoxy resin) or inside the machine when using Lowicryl resin. This latter resin is polymerized under a UV lamp, starting at -45oC, then gradually moving up to room temperature (Lowicryl HM20). At the end of the process hard plastic blocks are generated ready to be cut by ultramicrotomy.

Figure (16): Ultramicrotome.

ARTIFACTS AND TROUBLESHOOTING:

Artifacts are damage caused in specimen preparation and can be confused with specimen ultrastructure. Many artifacts are a result of mechanical or chemical action during sample preparation and some artifacts are due to irradiation by the electron beam during examination of the specimen in the microscope. Recognizing specimen damage is the initial step in preventing the same problem in future preparations and not reporting erroneous data.

Examination of nanoparticles by transmission electron microscopy (TEM) has become an important method for characterizing these particles.

1- Clumping of particles on grids often occurs if grids are not glow discharged before use to change a hydrophobic surface to a hydrophilic surface, or the solution of the particles is too concentrated. If negative staining is a part of the preparation, there should be attention to the choice of stain, concentration and pH.

2- Depending on the specimen, drying artifacts may change the characterization of the nanoparticles. Bacterial flagella often drop off if acidic stains are used and some structures can best be preserved by pre-fixation with 1% (vol/vol) aqueous glutaraldehyde prior to negative staining.

3- Artifacts in sections can be the result of poor ultramicrotomy skills or problems in embedding. Microtomy problems range from loose fittings with the microtome and specimen, too large block face, improper setup of the cutting arc and dirt or damaged areas on the knife edge. Poor dehydration and infiltration as well as improperly polymerized resins contribute to artifacts in sections.

4- Uranyl acetate is an important reagent in fixation and staining of biological specimens; however, it

can also be the source of significant artifacts if used inappropriately. En bloc staining with uranyl acetate extracts glycogen and use of uranyl stains in the presence of phosphate buffers results in needle-like crystals all over the specimen or grid. There are methods to remove these stain artifacts; but it is better not to allow the formation of these artifacts.

5- Post staining of grids with uranyl acetate followed by lead citrate is a common source of artifacts. One source of problems is old stains which often result in poor staining or require longer staining times which can result in stain precipitates due to drying. The correct pH of lead stains is 12 and this can best be achieved by using commercially prepared carbonate-free solutions of 1N NaOH. The quality of the water that is used to prepare stains and wash grids is also important. If the laboratory deionized water supply is not of the best quality, it may be necessary to purchase distilled or HPLC grade water just for preparing stains and staining grids.

6- A perplexing problem with glutaraldehyde and osmium fixation is the presence of electron dense precipitates in sections. The use of divalent cations can result in precipitates of proteins. Magnesium (Mg^{++}) is preferred to calcium (Ca^{++}) since it is a smaller molecule and at low concentrations does not precipitate proteins as readily. In some tissues this may be a problem if phosphate buffers are used with glutaraldehyde and osmium; however, this phenomenon has been observed with cacodylate buffer also. Osmium pepper precipitate can be removed by treating sections with 1% (wt/vol) periodic acid (freshly prepared) in water for 5-10 minutes followed by several washes in deionized water and then usual post staining. If the formation of the osmium pepper precipitate becomes a recurring problem with the specimen and fixation protocol used, there is a recommendation to eliminate the formation. Inclusion of 0.1 M glycine in buffer washes has been advocated for removing unbound aldehydes from specimens for immunolabel localization and in addition, inclusion of 0.5-1.0 % (vol/vol) dimethyl sulfoxide (DMSO) in buffer washes for cytochemical localizations has been used to rapidly remove unbound fixative. In the buffer washes after aldehyde fixation, add 0-5-1.0% (vol/vol) DMSO and finally add 0.1M (wt/vol) glycine to the final two buffer washes before any other procedures and the final osmication.

7- Scanning electron microscopy (SEM) has artifacts of charging which indicate that the specimen is not properly grounded and drying artifacts can result from the manner in which specimens are prepared. Critical point drying (CPD) and/or use of chemical critical point drying by use of hexamethyldisilazine (HMDS) eliminates drying artifacts while charging can be reduced by proper coating with heavy metals. Specimens with intricate shapes cannot always be adequately coated with gold or palladium or other heavy metals by sputter coating. Vapor coating with osmium or ruthenium vapors offers a simple way to coat areas of specimens that cannot be reached by sputter coating. In addition, care must be taken in choice of coating material such that there is no interference of peaks in electron spectroscopic analysis.

REFERENCES

1- Beisker W et al. (1987) Cytometry 8:235–239.
2- Cowen T et al. (1985) Histochemistry 82:205–208.
3- Mosiman VL et al. (1997) Cytometry 30:151–156.
4- Romijn, Herms J et al. (1999) J Histochem Cytochem 47:229–236.
5- Bancroft JD, Stevens A: Theory and Practice of Histological Techniques.4th Ed., Churchill-Livingstone, New York, 1995
6- Luna LG: Histopathologic Methods and Color Atlas of Special Stains and Tissue Artifacts.American HistoLabs, Inc., Gaithersburg, MD, 1992
7- R. F. E. Crang and K. L. Klomparens, "Artifacts in biological electron microscopy.", (Springer, New York) 233 pp. [2] B. Cao, H. Xu and C. Mao, Microsc. Res. Tech. 74(2011), 627. [3] B. Michen et al., Scientific Reports 5 (2015), 1.
8- N. Reid "Ultramicrotomy", ed. AM Glauert (North-Holland Publishing Co., New York) 300 pp. [5] J. Kuo, G. L. Husca, and LND Lucas, Stain Technol. 56(1981), 221.
9- E. A. Ellis, in "Electron Microscopy, 3rd Ed." ed. J Kuo (Humana Press, New York) p. 57. [7] E. S. Reynolds, J. Cell Biol. 17(1963), 208.
10- A. M. Glauert and P. R. Lewis, "Biological specimen preparation for transmission electron microscopy", ed. A. M. Glauert (Noth-Holland Publishing Co, New York) 194 pp. [9] E. A. Ellis and D. W. Anthony, Stain Technol. 54(1979), 282. [10] P. Echlin 2009. "Handbook of sample preparation for scanning electron microscopy and x-ray microanalysis", (Springer, New York) 330 pp.

Book Coordinator ; Mostafa Fathallah

General Directorate of Technical Education for Health