Histochemistry

Zoo-103, Unit V

By

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Histochemistry is a method of staining tissue that provides information concerning the presence and location of intracellular and extracellular macromolecules.

- Histochemistry is complementary to biochemical analysis of tissue homogenates, since histochemical techniques can give simultaneously biochemical and morphological information.

The general protocol used for histological and histochemical study can be divided into the following main parts:

- Sampling
- Fixation
- Dehydration, clarification
- Impregnation, embedding
- Sectioning (sections can be done with embedded or frozen organs)
- Adhesion of section on a slide
- Staining (a lot of staining methods can be used for histological as well as histochemical study)
- Dehydration; it is useless (and even wrong) in the case of mounting with a hydrophilic medium
- Mounting (can be done with hydrophobic or hydrophilic medium)
- Observation

TISSUE PREPARATION

In case of Tissue Dissection:

- fixating as quickly as possible
- washing tissues into an iso-osmotic physiological liquid

In case of cell culture:

- Empty the cell culture medium
- Place the fixative.
- Let the cell remain in contact with the fixative for several hours.

Fixation

- Fixation preserve the tissue morphology in a recognizable form,

Effects of Fixation:

- Immobilization of cell components
- Inhibition of cell autolysis
- Inhibition of putrefaction
- Tissue hardening
- Modification of tissue refraction index that permits observing them even before staining
- Effects on dye affinity

Different Fixative Types

- Denaturant fixatives are still called coagulant.

Coagulant Fixatives:

- Ethanol—Ethanol (70% to 100%) is mainly used to preserve mineral elements.
- Picric acid
- Mercury chloride

Noncoagulant Fixatives:

- Formaldehyde
- Osmium tetroxide (a very good cytological fixative that reacts with the lipids, more particularly phospholipids that belong to the cell membrane structure).
- Acetic acid (good fixative for the nucleus)

Fixative Mixtures:

- Alcohol–formalin ; Bouin's fluid; Carnoy's fluid

Different Fixative Types

Fixation Duration:

- Bouin's fluid: 24 to 48 h
- Carnoy's fluid: 4 h
- Formalin: Indefinitely

Methods for Chemical Fixation

- **Fixation by Immersion**. immerse the tissue fragment in the fixative as quickly as possible.
- **Fixation by Perfusion**. Perfusion is used for the fixation of tissues that are particularly fragile e.g. brain tissue
- **Fixation with Osmium Tetroxide Vapor** . It is essentially used to fix a blood smear. Quickly place the smear above the fixative for several seconds where the face of the slide with the smear is placed above the fixative

Methods for Physical Fixation

- ***Cryodessication:*** a fresh tissue is quickly frozen, then dried under vacuum at a very low temperature. Tissue water goes directly from the solid state to the gaseous state. The tissue fragment is then directly embedded in melted wax.
- ***Freezing–Dissolution:*** In freezing–dissolution a fresh tissue is quickly frozen, then the ice is dissolved with absolute ethanol. Embedding is then done in melted wax. In freezing microtome, the temperature of both blade and tissue is very low. In cryotome, the microtome is entirely contained in a cold enceinte, the "cryostat."
- ***Classic Fixation by Cold:*** In certain cases, fresh tissue is directly fixed by cold, then cut with a freezing microtome or a "cryotome." Freezing temperature is generally between –20°C and –40°C.

Methods for Physical Fixation

- ***Chemical and Cold Fixation***: Tissues that were fixed by chemical fixative may be cut without embedding by means of a freezing microtome or a cryotome.

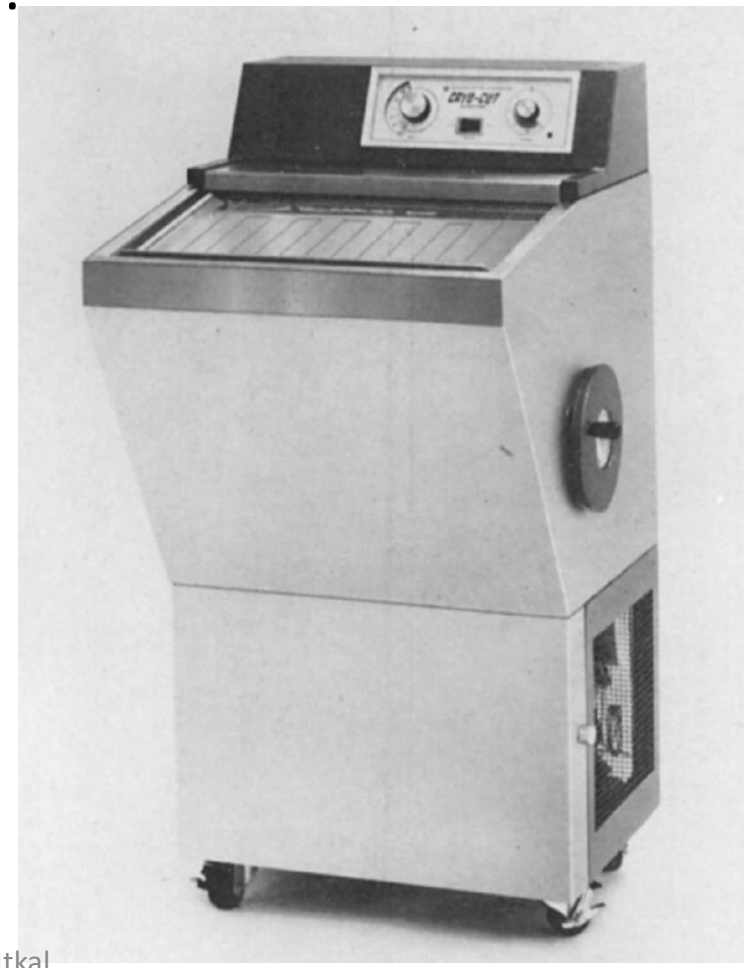
Fixed tissue and the cryostat

- For most diagnostic purposes in a routine laboratory, cryostat sections of unfixed tissue are suitable.

Freezing of fresh unfixed tissue

The fresh tissue should be frozen as rapidly as possible without creating freeze artifacts. Suitable techniques include:

- Liquefied nitrogen (–190°C).
- Isopentane (2-methylbutane) cooled by liquid nitrogen (–150°C).
- Dry ice (–70°C).
- Carbon dioxide gas (–70°C).
- Aerosol sprays (–50°C).
- Internal freezing shelf or bar.



Fixed tissue and the cryostat

Gum sucrose solution

Gum acacia	2 g
Sucrose	60 g
Distilled water	200 ml

Store at 4°C

Method

1. Fix fresh tissue block in formal calcium at 4°C for 18 hours.
2. Rinse in running water, or for a short time in distilled water if the tissue fragment is small or fragile, e.g. jejunal biopsy.
3. Blot dry.
4. Place tissue in the gum sucrose solution at 4°C for 18 hours or less with small fragments.
5. Blot dry.
6. Freeze tissue onto the block holder.

Following fixation, the block is frozen slowly to avoid damage caused by the rapid expansion of ice within the tissue. Freezing the block by standing it in the cryostat cabinet produces acceptable results for the majority of fixed tissue. The length of time required for this procedure limits its value as a diagnostic tool.

Fixed tissue and the cryostat

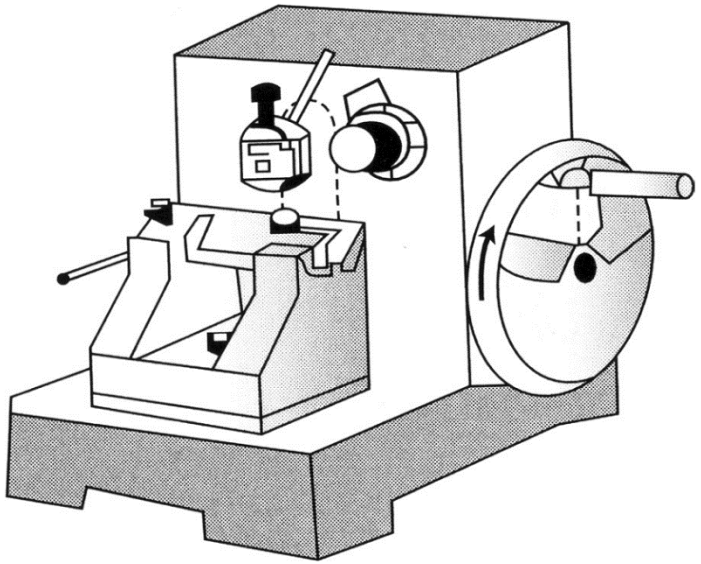
- For most diagnostic purposes in a routine laboratory, cryostat sections of unfixed tissue are suitable.

Embedding

- 1. **Paraffin Embedding**: tissue must first be dehydrated by ethanol (or acetone) before putting in wax.
- **Dehydration** of tissues preserved in Bouin's fluid or formalin is carried out by – Ethanol 70%, 4 h. Ethanol 100%, 2 × 4 h.
- **Clarification** of tissue (tissue fragments become transparent) is done by use of Butanol, 2 × 12 h
- **Paraffin impregnation** : Leave the tissue in melted paraffin for 4 to 12 h depending on the tissues: 4 h for liver, kidney, spleen, and lung, and 12 h for the other tissues.
- **Embedding**:The impregnated tissue is embedded in a paraffin block

Sections

- For Paraffin, Paraffin–Celloidin, or Gelatin–Paraffin Blocks:
- Blocks are put on the stage of a vertical microtome (Minot's microtome). The sections are usually retailed to obtain 4 to 7 µm thickness.



Vertical microtome.

Sections

- For Celloidin Sections
- Blocks are cut with a special horizontal microtome. Sectioning is done in ethanol 70%.
- For Sections for Plastic Waxes:
- knife is made of glass
- For Bone Sections:
- Sections are obtained by use of a tungsten carbide blade using Sections of wax-embedded bones
- For Frozen Sections:
- Cryostat is used. The piece of tissue is cooled. Then it is directly embedded in a wax that is liquid at laboratory temperature and solid at the low temperatures that are used for sections

Deparaffining and dehydration

- Before they can be stained by dye or by a histochemical reactive in aqueous solution, paraffin embedded sections must be cleared and hydrated.
- Dewaxing is done with a solvent. Then, hydration is done by putting slides in baths containing decreasing degrees of ethanol and finally water.

METHODS OF STAINING

- Use of dyes
- A dye is a molecule that empirically possesses two particular chemical groups: the chromophore, which gives the color, and another atomic group, the auxochrome, which is required to fix the dye molecules on the tissue-specific molecules.

The main chromophore groups are

- Azoic
- Azine
- Indamine or thiazine
- Nitro
- Kinonic form of aromatic molecules and naphtokinones

The higher the number of chromophoric groups, the more intense the coloration will be.

METHODS OF STAINING

- Acidic and Basic Dyes:
- In classic histology, it is recognized that acidic dyes are cytoplasmic and basic dyes are nuclear. However, this acid/base terminology is not linked to the pH of the dye solution, and it is independent of the acidic or basic nature of this solution.
- The distinction refers to the auxochrome, which is the part of the dye molecule that is required for tissue fixation.
- Eosin sodium salts, which are acidic dyes, generally have a basic pH.
- Basic dyes possess a –NH3+ cationic auxochrome. In contrast, acidic dyes possess an anionic auxochrome, such as –COO– or even SO3–. When a SO3– group is fixed on a basic dye, the latter is transformed in acidic dye (for example, acidic and basic fuchsin).
- When a staining substance does not have an auxochromic group, a mordant is useful. Iron and ammonium alum are often used as mordant

METHODS OF STAINING

- Chromosome Staining:
- by Acetocarmine Reagents
- Protocol

Acetocarmine, a few drops

Cover with a coverslip

Push slightly

Dry with paper filter

Seal

- Results—Chromosomes are red stained. Chromosomes can be violet stained by adding ferric chloride into the acetocarmine solution.

DEMONSTRATION OF CARBOHYDRATES

- **Simple carbohydrates**
  Monosaccharides
  glucose, mannose, galactose
  Oligosaccharides
  sucrose, maltose
  Polysaccharides
  glycogen, starch
  Glycoconjugates
  Connective tissue glycoconjugates
  proteoglycans
  hyaluronic acid
  Mucins
  neutral mucins
  sialomucins
  sulfomucins
  Other glycoproteins
  membrane proteins (receptors, cell adhesion molecules)
  blood group antigens
  Glycolipids
  cerebrosides
  gangliosides

DEMONSTRATION OF CARBOHYDRATES

Type	Location	Function	Associated pathological condition
Glycogen	Liver, skeletal muscle, cardiac muscle, hair follicles, cervical epithelium, etc.	Storage form of carbohydrate	Found in a wide range of malignancies – Ewing's sarcoma/PNET, rhabdomyosarcoma, seminoma, etc. Abnormal accumulation in tissues of patients with glycogen storage diseases
Proteoglycans and hyaluronic acid	Cartilage, heart valves, blood vessels, tendons, ligaments, extracellular matrices, and ubiquitously expressed on the membranes of many cell types	Support, lubrication, cell adhesion, etc.	Found in certain sarcomas – myxoid chondrosarcomas, myxoid liposarcomas, myxoid fibrous histiocytomas, etc. Abnormal accumulation in tissues of patients with mucopolysaccharidoses
Mucins	Epithelia of the gastrointestinal tract, respiratory tract, reproductive tract	Secreted mucins – lubrication and protection Membrane-bound mucins – cell adhesion and regulation of proliferation	Frequently found in adenocarcinomas of the gastrointestinal tract Aberrant or inappropriate expression of specific mucin types occurs frequently in the neoplastic process
Glycoproteins	Ubiquitously expressed on cell membranes Blood group antigens Secreted products such as peptide hormones and immunoglobulins	Multiple and diverse functions such as cell adhesion, immune recognition, regulation of receptor ligand binding, etc.	Aberrant expression of blood group antigens in various malignancies

DEMONSTRATION OF CARBOHYDRATES

The periodic acid-Schiff (PAS) technique

•Mechanism of the PAS technique:

the PAS technique is based upon the reactivity of free aldehyde groups within carbohydrates with the Schiff reagent to form a bright red/magenta end product.

- The initial step in the PAS technique is the oxidation of hydroxyl groups attached to adjacent carbon atoms (1,2-glycols) within the carbohydrate.
- The result is the formation of two free aldehyde groups and the cleavage of the adjoining carbon-tocarbon bond.
- The intensity of the color that develops following reaction with Schiff reagent is dependent upon the tissue concentrations of reactive glycol structures.

DEMONSTRATION OF CARBOHYDRATES

The periodic acid-Schiff (PAS) technique

PAS technique *(modified McManus 1946)*

Periodic acid solution

Periodic acid	1 g
Distilled water	100 ml

Preparation of Schiff reagent

Dissolve 1 g of basic fuchsin and 1.9 g of sodium metabisulfite ($Na_2S_2O_5$) in 100 ml of 0.15 M hydrochloric acid (HCl). Shake the solution at intervals or on a mechanical shaker for 2 hours. The solution should be clear and yellow to light brown in color. Add 500 mg of activated charcoal and shake for 1 to 2 minutes. Filter the solution through a No. 1 Whatman filter into a bottle. The filtered solution should be clear and colorless. If the solution is yellow, repeat the charcoal decolorization using a fresh lot of activated charcoal. Store at 4°C. Solution is stable for several months.

DEMONSTRATION OF CARBOHYDRATES

The periodic acid-Schiff (PAS) technique

Method

1. Dewax in xylene and rehydrate through graded ethanols to distilled water.
2. Oxidize with periodic acid for 5 minutes.
3. Rinse in several changes of distilled water.
4. Cover the sections with Schiff reagent for 15 minutes.
5. Rinse in running tap water for 5–10 minutes.
6. Stain the nuclei with hematoxylin. Differentiate and blue the sections.

DEMONSTRATION OF CARBOHYDRATES

The periodic acid-Schiff (PAS) technique

7. Dehydrate in graded ethanols and clear with xylene.
8. Coverslip.

Results

Glycogen, neutral/sialomucins	magenta
Various glycoproteins	magenta
Nuclei	blue

Notes

a. The intensity of stain is dependent to some extent on the length of treatment with the periodic acid and Schiff reagent. For basement membranes, a longer time in periodic acid (10 minutes) and Schiff reagent (20 minutes) may give better results.

DEMONSTRATION OF CARBOHYDRATES

The periodic acid-Schiff (PAS) technique

- **Notes**
- Fixatives containing glutaraldehyde should be avoided if tissues are to be stained with the PAS technique.
- Staining of glycolipids may be detected when frozen sections are used.

Demonstration of Proteins

Methods of demonstration

1. histophysical methods
2. amino acid histochemical methods
3. enzyme histochemical methods
4. immunocytochemical methods.

Demonstration of Proteins

Amino acid histochemical methods

These methods demonstrate the presence of some of the constituent amino acids rather than whole protein molecules. They are based upon the identification of exposed groupings and linkages within the amino acid molecules:

1. protein-bound amino groups, e.g. in lysine
2. phenyl groups. e.g. in tyrosine
3. disulfide and sulfhydryl linkages, e.g. in cystine and cysteine
4. indole groups, e.g. in tryptophan and tryptamine
5. guanidyl groups, e.g. in arginine.

Demonstration of Proteins

Protein-bound amino groups

These can be demonstrated by the ninhydrin-Schiff reaction. At neutral pH and 37°C, ninhydrin reacts with α-amino groups to produce aldehydes which can then be demonstrated using Schiff's reagent.

Demonstration of Proteins

Ninhydrin-Schiff method for amino-groups (Yasuma & Itchikawa 1953)

Fixation

Neutral buffered formalin; formaldehyde vapor (for freeze-dried tissue).

Sections

Paraffin, cryostat or freeze-dried.

Solutions

0.5 per cent ninhydrin in absolute alcohol.

Schiff's reagent

Method

1. Take sections to 70 per cent alcohol.
2. Treat with ninhydrin solution at 37°C overnight.
3. Wash in running tap water.
4. Treat in Schiff's reagent for 45 min.
5. Wash in running tap water.
6. Counterstain with an alum hematoxylin.
7. Wash in tap water; dehydrate through alcohols, clear in xylene and mount in DPX.

Demonstration of lipids

- Because a lot of lipids are soluble in organic solvents, the fixatives must not be of organic nature. Because of the organic solvent being used with paraffin embedding, *lipids must be treated to become insoluble before embedding*, or *use frozen sections.*

Demonstration of lipids

- *Fixation and Insolubilization:*
- *Protocol 1:*
- *Fixation into the mixture of [Potassium bichromate 5%, 80 mL; Formalin, 15 mL; Acetic acid, 5 mL;] for 12 hr*
- Wash with potassium bichromate 3%, 5 min followed by wash in Potassium bichromate 3%, 2 days at 60°C
- *Wash with tap water*
- *Frozen sections or paraffin embedding*

Demonstration of lipids

- *Fixation and Insolubilization:*
- *Protocol 2:*
- *Fix tissue with formalin–calcium, 6 to 12 h*
- *Treat with potassium bichromate 3%, 2 days at 60°C*
- *Frozen sections or paraffin embedding*

Demonstration of lipids

Standard Sudan black B method for fats and phospholipids

Fixation and sections

Cryostat sections post-fixed in formol-calcium; short fixed frozen sections; unfixed cryostat sections (preferred).

Method

1. Rinse sections in 70% ethanol.
2. Stain for up to 2 hours in saturated Sudan black B in 70% ethanol.
3. Rinse in 70% ethanol to remove excess surface dye, and wash in tap water.
4. Counterstain nuclei with Kernechtrot for 2–5 minutes.
5. Wash well and mount in glycerin jelly.

Demonstration of lipids

Standard Sudan black B method for fats and phospholipids

Results

The standard Sudan black procedure stains unsaturated esters and triglycerides *blue-black*. Some phospholipids appear *gray* and those in myelin exhibit a *bronze* dichroism in polarized light.

Bromination enhances the reaction of these lipids and in addition stains lecithin, free fatty acids and free cholesterol.

Demonstration of Nucleic Acid

General notes on nucleic acid demonstration

Fixation

In general terms, the nucleic acids are best preserved in alcoholic and acidic fixatives, a good example being Carnoy's fluid which contains both alcohol and glacial acetic acid. Formalin has only a limited reaction with DNA and RNA, but for routine work gives acceptable results. Low (4°C) temperature fixation in neutral buffered formalin has been shown to prevent DNA degradation by cell nucleases. which is of some importance when carrying out molecular biology studies (Tokuda et al. 1990).

Demonstration of Nucleic Acid

Demonstration of DNA by Feulgen and rossenbeck's nuclear reaction method

Fixation

Fixative—Carnoy's or Flemming's fluids are often recommended, but it is possible to use numerous other fixatives.

Fixative	Time (minutes)
Bouin	Unsuitable
Carnoy 6.3.1	8
Chrome-acetic	14
Flemming	16
Formaldehyde vapor	30–60
Formalin	8
Formol-sublimate	8
Helly	8
Newcomer	20
Regaud	14
Regaud-sublimate	8
Susa	18
Zenker	5
Zenker-formol	5

Demonstration of Nucleic Acid

Demonstration of DNA by Feulgen and rossenbeck's nuclear reaction method

Preparation of solutions

a. 1 M hydrochloric acid

Hydrochloric acid (conc.)	8.5 ml
Distilled water	91.5 ml

b. Schiff's reagent

c. Bisulfite solution

10 per cent potassium metabisulfite	5 ml
1 M hydrochloric acid	5 ml
Distilled water	90 ml

First the tissue is fixed in Carnoy fixative / or any fixative for the duration described in previous slide Table , followed by tissue block preparation in wax & tissue cutting

Demonstration of Nucleic Acid

Demonstration of DNA by Feulgen and rossenbeck's nuclear reaction method

Method

1. Bring all sections to water.
2. Rinse sections in 1 M HCl at room temperature.
3. Place sections in 1 M HCl at 60°C
4. Rinse in 1 M HCl at room temperature, 1 min.
5. Transfer sections to Schiff's reagent, 45 m
6. Rinse sections in bisulfite solution, 2 min.
7. Repeat wash in bisulfite solution, 2 min.
8. Repeat wash in bisulfite solution, 2 min.
9. Rinse well in distilled water.
10. Counterstain if required in 1 per cent light green, 2 min.
11. Wash in water.
12. Dehydrate through alcohols to xylene and mount.

Demonstration of Nucleic Acid

Demonstration of DNA by Feulgen and rossenbeck's nuclear reaction method

Results

DNA	*red-purple*
Cytoplasm	*green.*

Notes

a. The hydrolysis time is important (see Table 12.1), and the correct time for the fixative must be used.
b. The 1 M HCl should be preheated to 60°C.
c. Elias et al. (1972) use 5 M HCl at room temperature for hydrolysis. The hydrolysis time needs to be longer than at 60°C.
d. The post-Schiff bisulfite solution washing is optional and, in our experience, can be safely omitted.

Demonstration of Nucleic Acid

Demonstration of RNA by Methyl green-Pyronin method

Methyl green–pyronin method
(Pappenheim 1899; UNNA 1902 from Bancroft & Cook 1994)

Fixation

Carnoy preferred, but formalin acceptable.

Staining solution

2 per cent methyl green (chloroform washed)	9 ml
2 per cent pyronin Y	4 ml
Acetate buffer pH 4.8	23 ml
Glycerol	14 ml

Mix well before use.

Demonstration of Nucleic Acid

Demonstration of RNA by Methyl green-Pyronin method

Methyl green–pyronin method
(Pappenheim 1899; UNNA 1902 from Bancroft & Cook 1994)

Method

1. Take sections down to water.
2. Rinse in acetate buffer pH 4.8.
3. Place in staining solution for 25 min.
4. Rinse in buffer.
5. Blot dry.
6. Rinse in 93 per cent alcohol, then in absolute alcohols.
7. Rinse in xylene and mount in DPX.

Results

DNA	*green-blue*
RNA	*red*.

Notes

Alkaline phosphatase: the Gomori calcium method (1951, modified)

Fixation

Formol calcium at 4°C.

Sections

Pre-fixed cryostat preferred.

Preparation of incubating medium

2 per cent sodium β-glycerophosphate	2.5 ml
2 per cent sodium veronal	2.5 ml
2 per cent calcium nitrate	5.0 ml
1 per cent magnesium chloride	0.25 ml
Distilled water	1.25 ml

The final pH of the incubating medium should be between 9.0 and 9.4. The sodium veronal acts as the buffer vehicle and the magnesium ions as an enzyme activator.

Demonstration of Enzymes

Alkaline phosphatase: the Gomori calcium method (1951, modified)

Method

1. After suitable fixation, bring sections to water, incubate at 37°C for 25 min. to 6 hours. (See note b.)
2. Wash well in distilled water.
3. Repeat wash.
4. Treat sections with 2 per cent cobalt nitrate, 3 min.
5. Wash well in distilled water.
6. Repeat wash.
7. Immerse sections in 1 per cent ammonium sulfide, 2 min.
8. Wash well in distilled water.
9. Counterstain in 2 per cent methyl green (chloroform extracted).
10. Wash well in running tap water.
11. Mount in glycerin jelly.

Results

Alkaline phosphatase activity	*brownish-black*
Nuclei	green.

Alkaline phosphatase: azo dye coupling method using α naphthyl phosphate

Fixation

Formol-calcium at 4°C. Formol vapor.

Sections

Prefixed cryostat preferred.

Preparation of incubating medium

Sodium α naphthyl phosphate	10 mg
0.2 M Tris buffer (stock solution A) pH 10.0	10 ml
Diazonium salt (fast red TR)	10 mg

The final pH of the incubating medium should be between 9.0 and 9.4. The sodium α naphthyl phosphate is dissolved in the buffer, the diazonium salt is added and the solution well mixed. The solution is then filtered and used immediately.

Method

1. After fixation, bring sections to water, incubate at room temperature for 10–60 min.

Alkaline phosphatase: azo dye coupling method using α naphthyl phosphate

2. Wash in distilled water.
3. Counterstain in 2 per cent methyl green (chloroform extracted).
4. Wash in running tap water.
5. Mount in glycerin jelly.

Results

Alkaline phosphatase activity	*reddish-brown*
Nuclei	*green.*

Note

The final pH of the incubating solution is about 9.2. If paraffin sections are used, the incubating time will need to be extended.

Demonstration of Enzymes

Preparation of tissues for histology

(a) Fixation

First the tissue is placed in fixative and allowed to fix

(b) Dehydration, clearing and wax impregnation

Next, the tissue is trimmed and placed in a cassette (the two halves of which are shown here)

The holder is placed in a basket in the automatic processor

The processor transfers the tissue through a series of alcohol solutions of increasing strength, and then into a clearing agent (xylene) and finally into molten wax to complete the wax impregnation process

Fixation

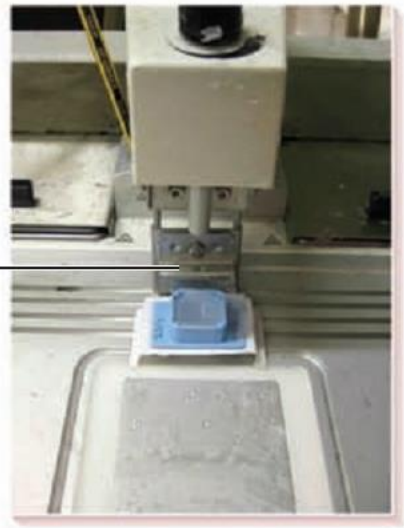
A chemical solution containing a fixative at pH 7.0 is added to the tissue (Fig. 1a). The most commonly used fixative is formaldehyde at a concentration of 4%. (Commonly, dilutions are made from a stock of Formalin, i.e., 37% or 40% formaldehyde.) Formaldehyde binds to and cross-links some proteins, and denatures others, but does not interact well with lipids. The overall effect is to harden the tissue and inactivate enzymes, preventing the tissue from degrading.

Dehydration

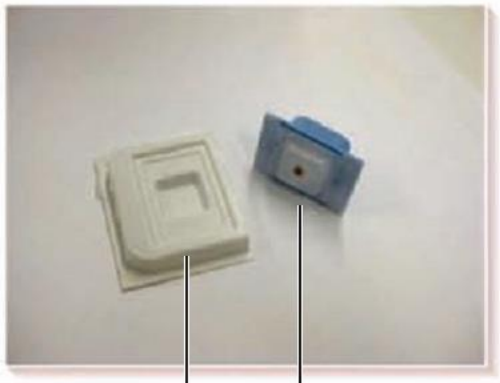
In order for sections to be cut, the tissue has to be embedded in wax. However, wax is not soluble in water. Therefore, the water in the tissue has to be removed and eventually replaced with a medium in which wax *is* soluble. This is achieved by, first, sequentially replacing the water with alcohol, placing the tissue in a series of solutions that contain increasing concentrations of alcohol, ending at 100% (Fig. 1b). This process is carried out gradually in order to minimize tissue damage. The tissue must then be 'cleared' before it can be embedded in wax.

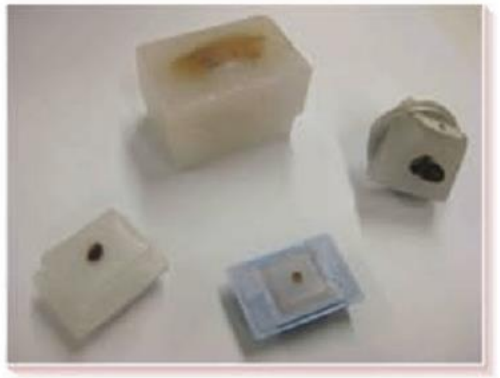
Preparation of tissues for histology

(c) Embedding



The tissue is transferred to a mould, and hot wax is dispensed into the mould

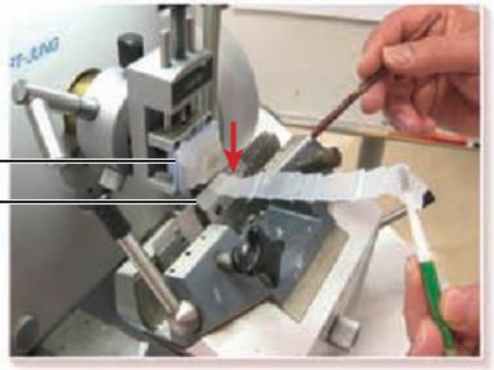




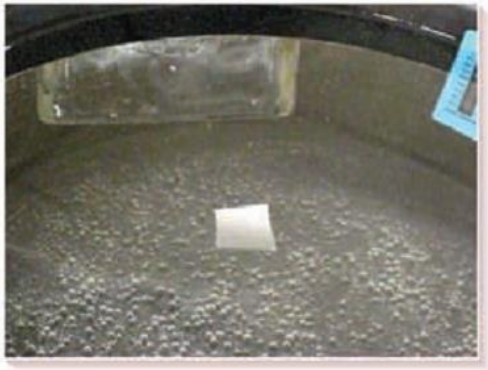
Blocks come in all shapes and sizes, depending on the size of the tissue

Preparation of tissues for histology

(d) Sectioning



The block is moved up and down (red arrow) and moved incrementally forward (toward the user) to cut sections. Serial sections emerge in a long ribbon, and are picked up with brushes



Single sections are picked up, floated on the surface of hot water, which removes the folds, and then transferred onto a glass slide

(e) Staining

The unstained section on the slide

The final slide after staining and mounting

Clearing

Next, the section is placed in an organic solvent such as xylene or toluene, which replaces the alcohol. Wax is not soluble in alcohol. The clearing agents are so-called, because the tissue often looks completely clear when it is immersed in clearing agent. Finally, the tissue is impregnated with hot wax (Fig. 1b), which is soluble in this type of organic solvent.

Embedding

The tissue is placed in warm paraffin wax in a mould (Fig. 1c). On subsequent cooling, the wax hardens, and tissue slices can now be cut.

Sectioning

Sections (slices) about 10 to 20 microns (μm) thick are cut using a microtome (Fig. 1d).

Mounting

The wax sections are laid onto a glass microscope slide (Fig. 1e).

Staining

To see detail, the components of the tissue have to be stained. However, the stains that are used are all aqueous. Therefore, the wax has to be dissolved and replaced with water (rehydration), for the stains to be able to penetrate the tissue section. The sections are therefore placed in decreasing concentrations of alcohol, ending up at 0% alcohol (water).

A number of different stains can be used but the most common is hematoxylin & eosin

Dehydration and mounting

The stained specimen is once again dehydrated, before placing it into mounting medium dissolved in xylene. Finally, a coverslip is placed on top of the sample to protect it, and the slide can be viewed on the microscope.

Other types of sectioning

Frozen sections

The tissue is rapidly frozen, fixed, and slices cut using a cryostat, before staining.

Semi-thin sections

The tissue is embedded in epoxy or acrylic resin, which has different properties to wax, and allows thinner sections (less than 2 µm) to be cut.

Enzyme histochemistry

- Enzyme histochemistry is the localization of enzymes in tissue by their specific action on a substrate

Differential diagnosis of tumours (*L*)

o-Diphenol oxidase[1] (EC 1.10.3.1) in melanoma

Acid phosphatase (EC 3.1.3.2) in prostatic carcinoma* and osteoclastoma

Alkaline phosphatase (EC 3.1.3.1) in adenocarcinoma of the lung*, seminoma of the testis*, and bone-forming tumours (osteosarcoma)

Leucine aminopeptidase (EC 3.4.1.1) in tumours of the stomach* bile ducts*, kidney*, mammary gland*, and fibrosarcoma*. Its presence in lymph node metastases strongly suggests carcinoma of stomach or bile duct*

ATPase (EC 3.6.1.3) in reticulum cell sarcoma (versus anaplastic carcinoma*)

Glycerolphosphate dehydrogenase (EC 1.1.99.5) in endocrine polypeptide (APUD) tumours

Characterization of tumours for prognosis (*Q*)

Level of carboxylesterase (EC 3.1.1.1)[2] in squamous carcinoma of lung; the lower the esterase activity, the worse the prognosis*

Level of alkaline phosphatase gives some indication of the prognosis of fibrosarcoma*

Characterization of non-neoplastic lesions (*L*, *Q*)

ATPase, glycogen phosphorylase (EC 2.4.1.1) reduced NAD dehydrogenase[3] (EC 1.6.99.3) and acid phosphatase in various skeletal muscle diseases

Jejunal acid phosphatase in coeliac disease (Wall *et al*, 1970)

Reduced NAD dehydrogenase and carboxylesterase (Garret and Howard, 1969) for demonstrating neurones in Hirschsprung's disease

Glycogen phosphorylases and glucose 6-phosphatase (EC 3.1.3.9) in glycogen storage diseases; also acid phosphatase in Pompe's disease (Lake, 1970)

Application of enzyme histochemistry to diagnostic pathology

Factors that Influence Enzyme demonstration

1. Enzymes are removed or destroyed by fixation, while others are sensitive to freezing and thawing, so compromises have to be made.
2. Non-optimal substrate: sometimes optimal substrate concentration can't be obtained because of poor substrate solubility.
3. Non-optimal temperature: there is an optimal temperature for enzyme activity, and sometimes, especially in azo dye simultaneous couple techniques, a different temperature must be used. Enzyme activity is usually destroyed at temperatures. greater than 56°C .
4. Non-optimal pH: most enzymes are best demonstrated at a pH near 7.0 , however, there are exceptions, as with acid and alkaline phosphatases.
5. Inhibitors: an excess of diazonium salts in the substrate, fixatives, heat and some metallic ion may decrease or completely abolish enzyme activity.