CHAPTER 6

STAINING

The sections, as they are prepared, are colourless and different components cannot be appreciated. Staining them by different coloured dyes, having affinities of specific components of tissues, makes identification and study of their morphology possible.

Certain terminologies used in the following account are given below.

**Basophilic**
Substances stained with basic dyes

**Acidophilic**
Substances stained by acid dyes

**Vital staining**
Staining of structures in living cells, either in the body (in vivo) or in a laboratory preparation (in vitro). e.g. Janus green is taken up by living cells and stains the mitochondria.

**Metachromatic staining**

There are certain basic dyes belonging to aniline group that will differentiate particular tissue components by staining them a different color to that of original dye. The phenomenon is known as metachromasia. The tissue element reacting in this manner are said to be exhibiting metachromasia.

The generally accepted explanation of this phenomenon is that change in color is due to polymerization.
Sulfated substances are highly metachromatic e.g. Mast cell granules.
These contain Heparin which is highly sulfated.
Some of the common metachromatic dyes are:

- Methylene blue
- Methyl violet
- Thionin
- Crystal violet
- Toluidine blue

Thionin and toluidine blue dyes are commonly used for quick staining of frozen selection using their metachromatic property to stain nucleus and cytoplasm differently.

Tissue components often demonstrated by metachromatic stains:

- Amyloid material
- Mast cell granules
- Mucin
- Cartilage

**Direct staining**

Application of simple dye to stain the tissue in varying shades of colours.

**Indirect staining**

It means use of mordant to facilitate a particular staining method or the use of accentuator to improve either the selectivity or the intensity of stain.

**Progressive staining**

Stain applied to the tissue in strict sequence and for specific times. The stain is not washed out or decolorised because there is no overstaining of tissue constituents. Staining is controlled by frequent observation under microscope.
**Regressive staining**

Tissue is first overstained and then the excess stain is removed from all but the structures to be demonstrated. This process is called differentiation and should always be controlled under microscope.

**Decolourization**

Partial or complete removal of stain from tissue sections. When the colour is removed selectively (usually with microscopic control) it is called differentiation. In case decolourization is to restain the selection with some other stain, acid alcohol treatment is the method of choice.

**Differentiation**

In regressive staining differentiation is the removal of washing out of the excess stain until the colour is retained only in the tissue components to be studies.

**Impregnation**

It is the deposition of salts of heavy metals on or around cells, tissue constituents etc. It has followed characteristics

1. Structures demonstrated are opaque and black
2. The colouring matter is particulate
3. The deposit is on or around but not in the element so demonstrated.

**Histochemical staining**

Staining which is used to indicate the chemical composition of the tissue or cellular elements.
**Counter stains**

A counter stain is the application to the original stain, usually nuclear, or one or more dyes that by contrast will bring out heavy counterstain is to be avoided least it mask the nuclear stain. It can be done either by using dilute stain or cutting down the staining time. Some counterstains which are acidic may lighten or remove the nuclear stains.

**Mordants**

Substance that causes certain staining reactions to take place by forming a link between the tissue and the stain. The link is referred as lack. Without it, dye is not capable of binding to and staining the tissue.

e.g. Ammonium and Potassium alum for hematoxylin.

**Accentuators**

These are substances that causes an increase in the selectively or in the staining power of dye. Thus they lead to more intense staining.

e.g. Phenol in Carbol fuchsin, KOH in Mehtylene blue.

**Leuco compounds**

Conversion of a dye into a colourless compound by the destruction of its chromophore. Prefix leuco is applied to it, e.g. leucofuchsin used in PAS stain.

**Dyes used in staining**

Dyes are classified in various ways:

<table>
<thead>
<tr>
<th>According to source</th>
<th>Affinity to tissues</th>
<th>Chemical compos</th>
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</thead>
<tbody>
<tr>
<td>Natural</td>
<td>Acidophilic</td>
<td>Thiazines</td>
</tr>
<tr>
<td>Synthetic</td>
<td>Basophilic</td>
<td>Rosailins</td>
</tr>
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</table>
Synthetic dye have greater staining capacity must greater spectrum of colours.

Natural dyes

These are very few in numbers. They are mainly low in common use.

1. **Haemotoxylin**: This is the most popular dye used as a nuclear stain. It is derived from the long tree mainly found in Mexico. It develops staining properly after oxidation. It is a weak dye and to make it give sharp stain a mordant is needed

2. **Carmine**: It is a scarlet dye made from the ground bodies of cochineal beetles.

Synthetic dyes

Most of these are in Aniline base and derived from coal tar. These aniline dyes offer wide range of colour and action. These aniline dyes offer wide range of colour and action. Chemical compostion may be basic, acidic, amphoteric (neutral). According to these characters stain different components of tissue.

**Basic dyes**

These are cationic dyes and stain nuclei, basophilic granules or bacteria.

**Acidic dyes**

These are anionic dyes and stain mainly cytoplasm, eosinophilic granules.
Theories of staining:

Physical theories:
1. Simple solubility e.g. Fat stains are effective because the stain is more soluble in fat than in 70% alcohol.
2. Absorption: This is a property by which a large body attracts to itself minute particles from a surrounding medium.

Chemical theories
It is generally true that acid dyes stain basic elements (Cytoplasm) and basic dyes stain acidophilic material (nucleus) however this far from being complete truth, Indeed hematoxylin, which is an acid dye, does nto stain the cytoplasm, but (in the presence of mordant) is one of the most widely used nuclear stains.

Staining of paraffin section
The most common method of histological study is to prepare thin sections (3-5 micron) from paraffin embedded tissues. These are then suitably stained and mounted in a medium of proper refractive index for study and strong. Commonest mounts used are resinous substances of refractive index close to that of glass. These are soluble in xylol. Hence sections are dehydrated and cleared in xylol and mounted. Mounting in aqueous mounting media is done directly after staining for sections which cannot be subjected to dehydrating and clearing agents.

The basic steps in staining and mounting paraffin sections are as follows:
1. Deparaffinisation
2. Hydration
3. Removal of mercury pigments wherever needed
4. Staining
5. Dehydration and clearing
6. Mounting

1. Deparaffinisation

Removal of wax is done with xylol. It is essential to remove the wax completely, otherwise subsequent stages will not be possible. At least 2 to 3 changes in xylol are given for suitable length of time. Sections of this stage should appear clear and transparent. Presence of any patches indicates the presence of wax and sections should be kept longer in the xylol.

2. Hydration

Most of the stains used are aqueous or dilute alcoholic solutions. Hence it is essential to bring the section to what before the stains are applied. The hydration is done with graded alcohol for higher concentration to lower concentration. Alcohol and acetone are miscible with xylol. First change is made to absolute alcohol or acetone followed by 90, 70% alcohol and finally distilled water.

Sections now should appear opaque. Presence of any clear areas are indicative of the presence of xylol. To remove this xylol, sections should be returned to absolute alcohol and rehydrated.
3. Removal of mercury pigments wherever needed

In case mercury containing fixatives e.g. Zenker, Susa etc are used, mercury pigments are precipitated on the sections. It has to be removed before staining is done. This is brought about by treatment with iodine solutions which changes mercury to an iodine compound. This in turn is converted to tetrathionate by thiosulphate, which is readily soluble in water, the slides are placed in running water to wash out all extraneous chemicals.

4. Staining

Various staining procedures are applied from this hydrates stage. The most common stain applied for histological study is Haemotoxylin and Eosin. Various types of haemotoxylin formulations are used. (See appendix.). Different types of staining procedures used are given in the appendix.

Certain of the stains use strong chemicals e.g. ammonia. Sections tend to float off the slides in such stains. This can be prevented by coating the selections by a thin layers of celloidin. For this sections are returned to absolute alcohol and then dipped in a dilute solution of celloidin and finally hardened in 70% alcohol. (For details see appendix).

5. Dehydration and clearing

Dehydration is done in graded alcohols or acetones from 70% to absolute alcohol or acetone. Dehydrating alcohol and acetones can remove some of the stains. Time has to be suitably modified to minimize fading of stains.
Since alcohol and acetone are miscible in xylol, it is used for clearing the sections. Any sections from which water has not been completely removed would give a milky appearance after the first xylol. Such sections should be returned to abs. alcohol and the process repeated. Mounting is done after 2nd or 3rd xylol.

6. Coverslipping and mounting

Make quite sure that the sections are quite clear. Do not let the section go dry before mounting

1. Hold the slide between the thumb and the forefinger of one hand and wipe with a clean cloth both ends of the slides. Look for the engraved number to make sure the side the sections is present.

2. Clean carefully around the section and lay on a clean blotting paper with section uppermost along with appropriate coverslip which has already been polished.

3. Place a drop of mountant on the slide over coverslip. Amount of mountant should be just enough. Invert the slide over the coverslip and lower it so that it just adheres to the cover slip quickly turn the slide over the lay it on a flat surface to allow the mountant to spread. Do not press or push the slide at all.

4. After the mountant has spread to the edge of the coverslip wipe around it for neatness. If proper care has been taken there should be no air bubbles. If many are present, slide should be returned to the
xylol to remove the coverslip. It will slip off and remounting is done.

No attempt should be made to pull the coverslip. Slight warming of the slide from below will make the small air bubbles to escape from the slide of the coverslip.

5. Coverslip should be in the center of the slide with neatly written label on one slide.

A good knowledge of various mountants and the coverslips is necessary for proper selection of the procedure.

**Mountants**

Histological sections which need to be examined for any length of time or to be stored, must be mounted under a cover-slip.

There are two types of mounting media:

1. Aqueous media - Used for material which is unstained, stained for fat, or mechanically stained.
2. Resinous media - For routine staining.

**Aqueous media**

There are used for mounting sections from distilled water when the stains would be decolorised or removed by alcohol and xylene, as would be the case with most of fat stains (Sudan methods). Some stains, e.g. methyl violent, tend to diffuse into medium after mounting. This can be avoided by using Highman's medium. Aqueous mountains require addition of
bacteriostatic agents such as phenol, crystal of thymol or sodium merthiolate to prevent the growth of fungi.

**Permanent seal** - After mounting the cover slip can be ringed by clear nail polish for storage.

Following are some of the commonly used aqueous mounting media: For formulation see the appendix.

1. Apathy's medium (R.I- 1.52)
   A very useful medium for mounting sections for fluorescent microscopy.
2. Farrant's medium (R.I. 1.43)
   Recommended for fat stains.
3. Glycerine jelly (R.I. 1.47)
   An excellent routine mountant for fat stains.
4. Highman's medium (R.I. 1.52)
   Recommended with the metachroamtic dyes especially methyl violent.

**Resinous mounting media**

Natural or synthetic resins dissolved in benzene, toluene or xylene. These are purchase readymade. In case they become too viscous they may have to be diluted with xylene. Following are some of these media.

1. Canada balsam - Natural resin (R.I. - 1.52)
It is used as 60% resin by weight in xylene. H.&E stained slides are fairly well preserved but basic aniline dyes tend to fade and prussian blue is slowly bleached. Slides take few months to dry.

2. D.P.X. (R.I. 1.52)
Polystyrene resin dissolved in xylene as a 20% solution. It is most commonly used.

3. There are many other synthetic resins sold under various trade names e.g. Coverbond (R.I. 1.53), H.S.R. (Harlew synthetic Resin), Histoclad (R.I. - 1.54), Permound (r.I. 1.54), Pro-Texx (R.I. 1.495).

Criteria of acceptable mounting media

1. Refractive index should be as close as possible to that of glass i.e. 1.5.
2. It should not cause stain to diffuse or fade.
3. It should be crack or appear granular on setting.
4. It should be dry to a nonsticky consistency and harden relatively quickly.
5. It should not shrink back from edge of cover-glass.
6. It should be free flowing and free bubbles.

Cover glasses used in histopathology

Care has to be exercised in selecting cover glasses for mounting, these are available in variable sizes and thickness and are supplied usually in 10 gm packings.
Following sizes are commonly available

22 x 22 mm  25 x 50mm
22 x 30 mm  Circular
22 x 40 mm

Cover glass should preferably be the No. 1 thickness (0.13 - 0.16 mm), but never more than No. 1 ½ thickness (0.16 - 0.19 mm).

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Haematoxylin and Eosin staining

Procedure

♦  Deparaffinize in hot air oven.

♦  Hydrate the section.
   i)  3 dips in xylene (2 Min. each)
   ii) 3 dips in acetone / alcohol (2 Min. each)
   iii) In running tap water for 5 Minutes.

♦  Mayer's haemotoxylin for 15 minutes.

♦  Wash in running tap water for 20 minutes

♦  Counter stain with eosin for 2 minutes

♦  Dehydrate the section in 95% and absolute alcohol/ acetone 2 changes (2minutes each).

♦  Clear in xylene 3 changes (2 minutes each)

♦  Mount in DPX
Results

Nucleus - blue
Cytoplasm and background - pink

Haematoxylin

Haematoxylin s supplied has no staining properties until it has been ripened by oxidation into haematin. This ripening is achieved by two methods:

1. Exposure of prepared solutions to the air for periods upto 6-8 weeks, preferably in sunlight or,
2. Addition of an oxidizing agent such as sodium iodate, potassium permaganate or mercuric oxide.

In this ripening process Haemotoxylin (C16 H1 & O6) loses two hydrogen atoms to become Haematin (C16 H12).

Sufficient Haematoxylin should be left unoxidized in solution, so that natural oxidation can continue. It prolongs shelf life of the stain.

Blueing

Alum Haematoxylin stains nuclei and red color which is converted to blue black color, when the section is washed in weak alkali. Tap water is usually alkaline enough to produce this color change.

Following may be used for rapid blueing of the sections.

1. 1% Lithium carbonate.
2. 2% Ammonia (Ammonia Water).
3. Scott's water

   Sod. or Pot. Carbonate  2 to 3 gm
   Magnesium sulphate  20 gm
   Dist. water  1000 ml

There are many formulations for preparing haemotoxylin stains. Use of many is a matter of personal preference of wether progressive or regressive staining is being used. In situations where haemotoxylin staining is followed by acidic stains, Ion haemotoxylin is preferred as it resists decolourisation by these counter stains. Various formulations differ mainly in regards to mordant and the shorter oxidiser used.

Following are the various formulations commonly used.

1. Harris's haemotoxylin - used for regressive staining and preferred in cytology.
2. Mayer's haemotoxylin - Most commonly used in routine work. Progressive staining is applied.
3. Iron haemotoxylin - should be used in situations where counterstains have strongly acidic character.
4. Phosphotungstic acid haemomatoxylin (PTAH) - This is exclusively used in some special stains for neurological fibers, muscle straitions and fibrin.
5. Others - Ehrlich's and Delafied haematoxylin. These may be used in place of Mayer's depending on perference. No chemical oxidiser is used, but the solution is kept in sunlight for 6 to 8 weeks for natural oxidation to take place.
While preparing the dye solutions care has to be taken in the amount of the chemicals added and the sequence of their addition. Follow the direction strictly.

**Some basic rules for staining**

1. Keep stains and solutions covered when not in use.
2. After the slides are removed from oven these should be cooled before being put in xylene.
3. Filter stains before use.
4. Once the slides have been put in the xylene to remove paraffin they should not be allowed to dry out. Particular care must be taken not to let the sections dry at the time of mounting as the xylene easily evaporates and if the section dried before mounting preparation would become useless.
5. Care should be taken that level of any solution used during staining is such as to cover the slides.
6. Drain the slides well and blot the bottom on filter paper before putting into the next solution. This is particularly necessary in transferring from 95% of Abs. alcohol and Abs. alcohol in xylol.
7. Xylol used to remove paraffin should not get mixed up with the clearing xylol. It also should be frequently changed as it tends to get saturated.
8. If for blueing an alkali e.g ammonia has been used, it should be well washed out. Failure to do that will lead to disagreeably hazy blue colour of nuclei.

**Causes of poor quality of staining**

1. Poor or inadequate fixation of tissue.
2. Over or under-ripened Haematoxylin.
3. Overused or worked out Haematoxylin.
4. Over or under differentiation of haematoxylin
5. Insufficient blueing following differentiation.
6. Failure to wash blueing agent out of section before counter staining with eosin (especially when ammonia is used).
7. Insufficient differentiation of eosin during washing or dehydration.
8. Insufficient dehydration and clearing of sections.