Principles of Tissue Demonstration

Objectives

At the end of the lecture you must be able to:

- Explain the aim of staining
- Discuss the different methods of staining
- Explain the structure of dyes
- Discuss histological classification of dyes
- Explain functions for the different types of non-dye constituents in staining solutions
- Describe how dyes bind to tissue
- Describe methods of producing silver deposits during the silver Impregnation technique

Aim of Staining

- To identify different tissue components by their color reaction.
- The stains are essentially a means of adding contrast to tissue components.
- Cytoplasm has about the same refractive index as glass.

Methods of Staining

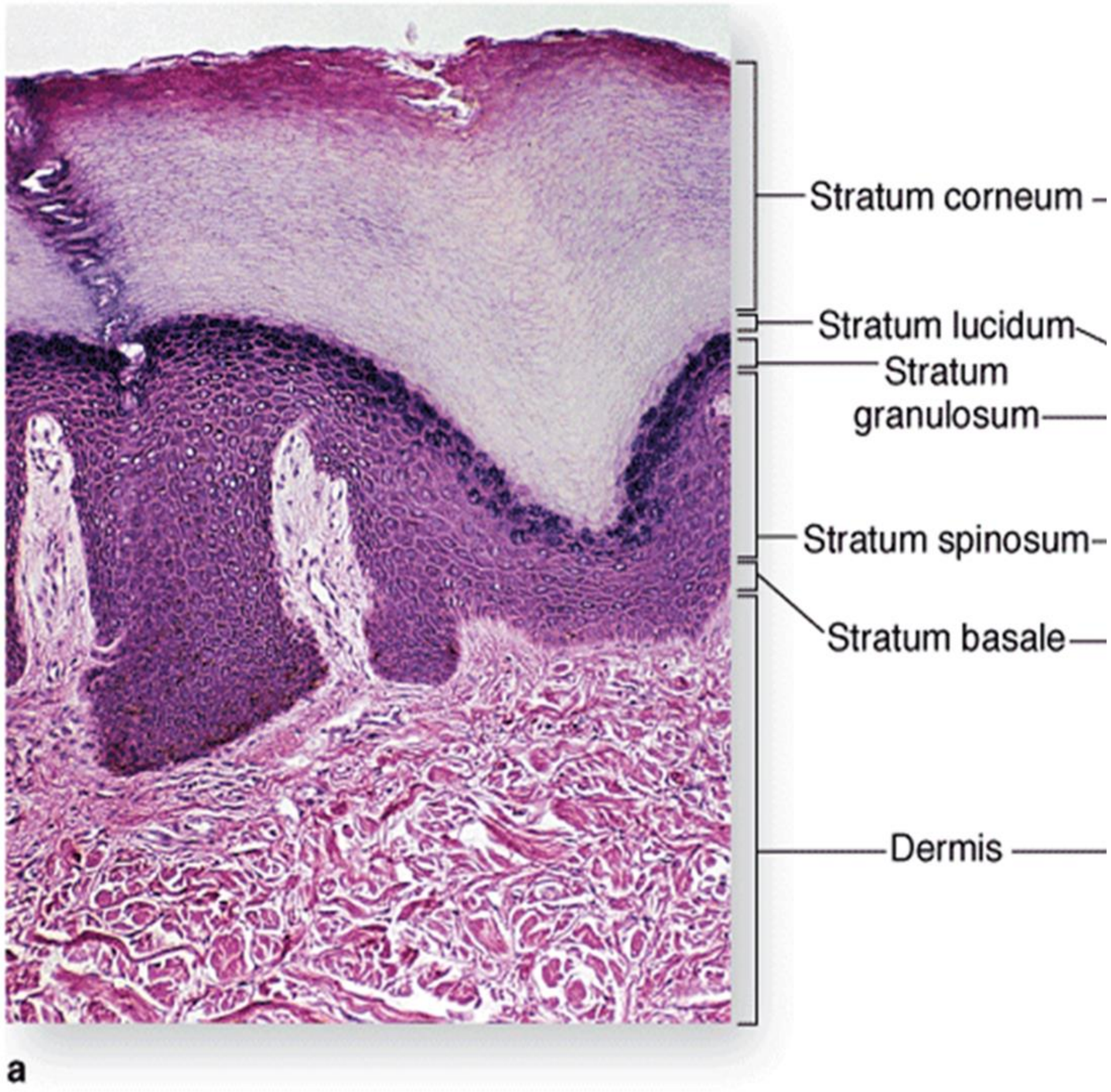
- Vital staining
- Routine staining
- Special staining
- Direct staining
- Indirect staining
- Progressive staining
- Regressive staining
- Impregnation

Vital Staining

- Vital stains are stains applied to living tissue by injection of staining solution into some parts of an animal body or by mixing the stain with living cells.
  - e.g. Janus green is taken up by living cells and stains the mitochondria.
- Primarily used for research purposes.

Routine Staining

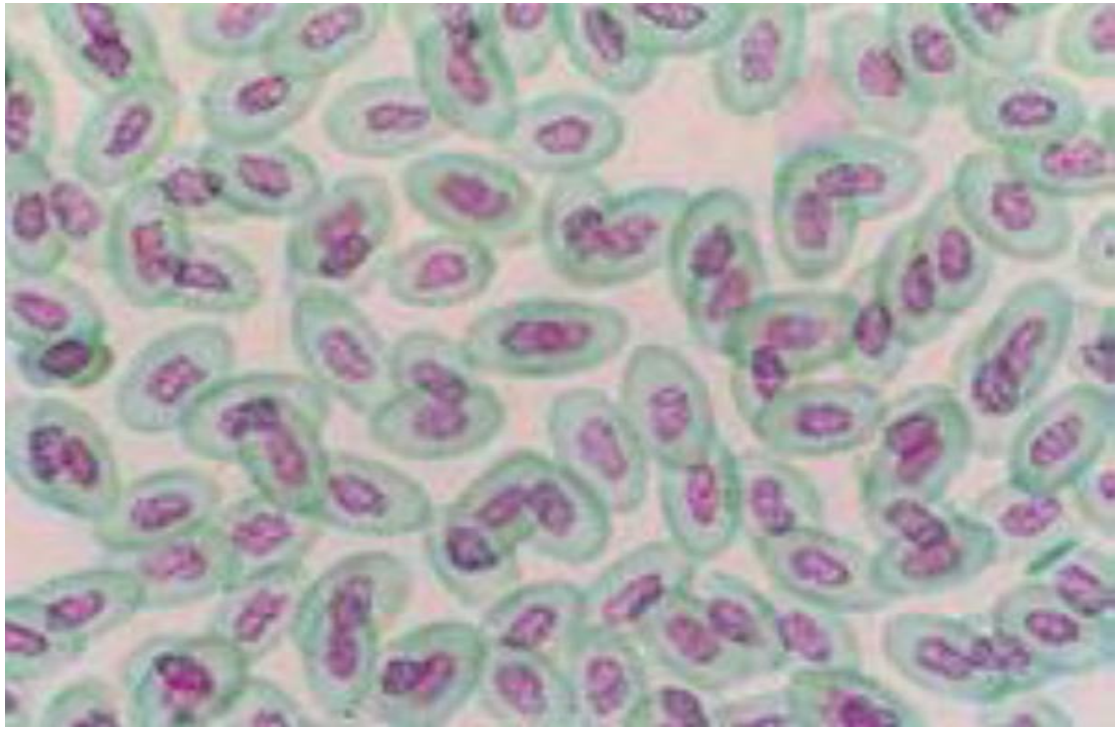
- A routine stain is one that stains the various tissue elements with little differentiation except between nuclei and cytoplasm, e.g. Hematoxylin and Eosin stain.



Source: Mescher AL: *Junqueira's Basic Histology: Text and Atlas, 12th Edition*: http://www.accessmedicine.com

Special (Selective) Staining

- Demonstrate special features of the tissue, such as bacteria, fungi, or particular cell products. e.g. Feulgen stain for DNA.



Feulgen Stain

DNA………Red-Purple
Cytoplasm…..Green

Indirect Staining

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

Direct Staining

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

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.
  - Differentiation is removal or washing out of excess stain until the colour is retained only by tissue component that are able to be studied.

- Can be done with acid alcohol or ethyl alcohol.

Progressive Staining

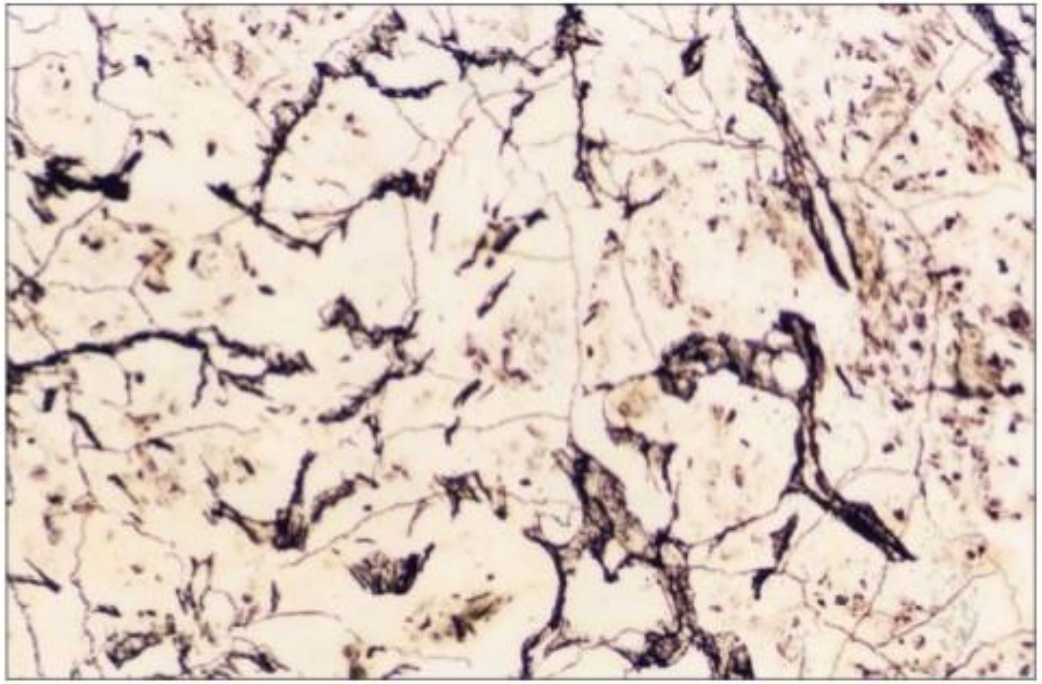
- Stain applied to the tissue in strict sequence and for specific times.
- The stain is not washed out or decolorized because there is no overstaining of tissue constituents.
- Staining is controlled by frequent observation under microscope.

Impregnation

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

- The structures so demonstrated are usually rendered opaque. The only colours possible are shades of brown or black.

Gomorri's Silver Impregnation Staining Technique for Reticulin Fibers



Reticular fibres : Black or dark brown

Structure of Dyes

- Dyes are coloured organic compounds that can selectively bind to tissues.

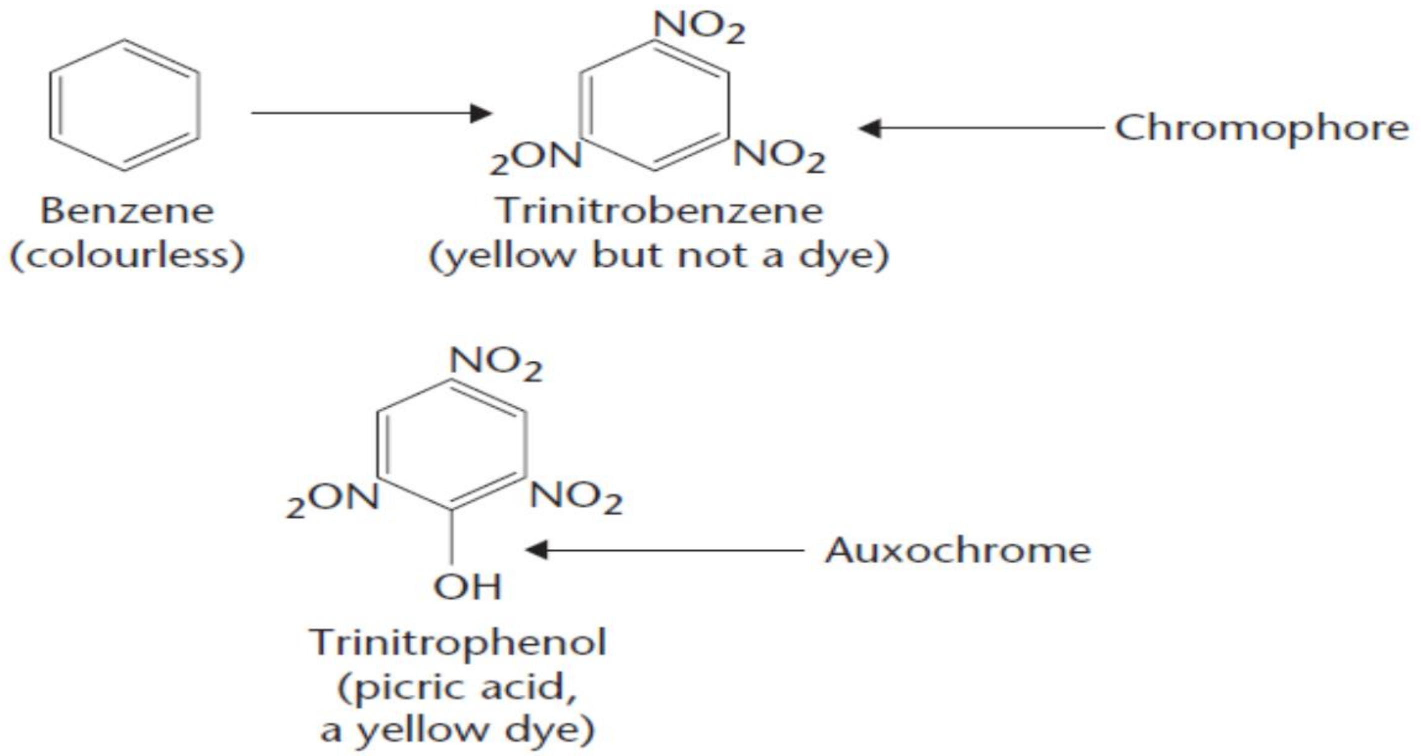


Figure 6.6
Conversion of benzene into a dye by the addition of a chromophore and an auxochrome

Histological Classification Of Dyes

- In histology it is often more useful to classify dyes by their action on tissues and hence their uses in histology.
    - Basic dyes
    - Acidic dyes
    - Neutral dyes
    - Amphoteric dyes
    - Natural dyes
    - Metachromatic dyes

Basic Dyes

- Are cationic and will stain anionic or acidic materials such as carboxylates, sulphates and phosphates.

- Most are used as nuclear stains.

- Acidic substances that stain with basic dyes are termed ***basophilic (e.g. DNA, RNA, Ribosomes)***.

    e.g. Haematoxylin, Crystal violet, methylene blue, safranin, basic fuschin

Acidic dyes

- Are anionic and will stain cationic or basic groups in tissues such as amino groups.

- Most are used to stain proteins in the cytoplasm and connective tissues.

- Substances that stain with acid dyes are called ***acidophilic (e.g. proteins, collagen, cytoplasm)***.

  e.g. Nigrosine, picric acid, eosin, acid fuschin, india ink etc

Neutral Dyes

- Are compounds of basic and acid dyes.

- Such dye complexes will stain both nucleus and cytoplasm from a single dye bath.

  - E.g. Romanowsky stains which include: Giemsa, Leishman and Wright's stains

Amphoteric Dyes

- Have both anionic and cationic groups, but these are on the same ion.
- Such dyes can stain either the nucleus or the cytoplasm if conditions are appropriate.

Natural Dyes

- Dye substances extracted from natural sources.

✓ E.g. haematoxylin, carmine, orcein and litmus.

Metachromatic Dyes

- The term ***metachromasia*** is used when a dye stains a tissue component a different colour to the dye solution.
  - E.g. Toluidine blue is a strong basic blue dye that stains nuclei a deep blue colour; however, it will also stain mast cell granules a pink colour.
- This colour shift that occurs is called metachromasia, whilst the usual blue staining is called ***orthochromasia***.

Non-dye Constituents of Staining Solutions

- Most staining solutions contain other components to improve the staining.
    - Mordants –metal salts that help some dyes bind to tissue
    - Trapping agents
    - Accentuators e.g. potassium hydroxide in Löffler's methylene blue
    - Accelerators e.g. hypnotic drugs e.g. barbiturates or chloral hydrate.

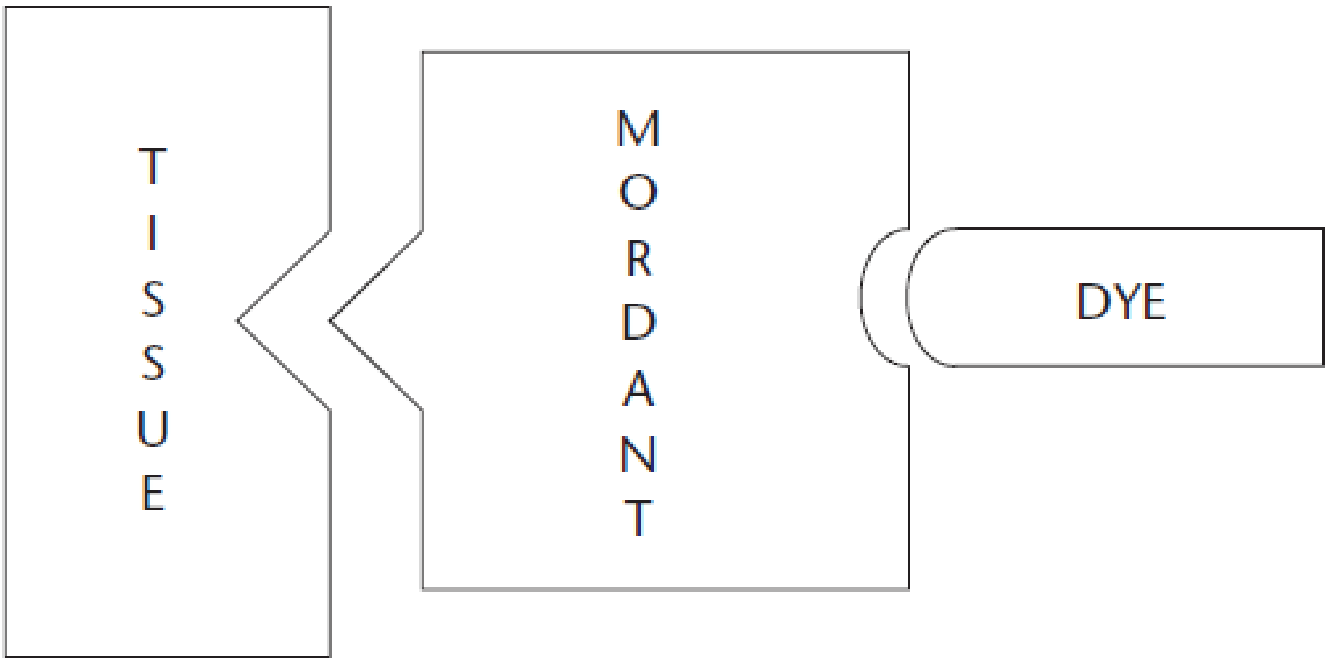


Figure 6.9
Mordanting. The dye can only bind strongly to the tissue when the mordant acts as a link between the two

Theory of Staining

- Stain uptake is often due to dye-tissue or reagent-tissue affinities.
- Affinity describes the tendency of a stain to transfer from solution onto a section or those attractive forces thought to bind dye to tissue.
- The binding of stains to tissues is a chemical bonding.

Factors Contributing To Dye-tissue Affinities

- Reagent-tissue interactions
- Solvent-solvent interactions
- Stain-stain interactions
- Solubility.

Reagent-tissue interactions

Coulombic attractions (ionic bonds)

- Arise from electrostatic attractions of unlike ions e.g. the colored cations of basic dyes and tissue structures rich in anions such as phosphated DNA, or sulfated muco-substances.

Van der Waals' forces

- Include intermolecular attractions i.e. dipole-dipole, dipole-induced dipole.
- These forces are polar attractions, effective over a short distance.
- E.g. staining of elastic fibers by orcein
  - Orcein is a large molecular weight dye
    - It has stronger dipoles
  - Elastin is hydrophobic protein
    - Has many polarisable amino acids

Covalent bonding

- In contrast to ionic bonding, covalent bonding involves sharing of electrons
- Are very strong bonds and are not easily broken once formed.
- This type of bond are involved in the fundamental reaction between a dye and a mordant.
- They are important in some histochemical techniques
  - e.g. periodic acid–Schiff, and in the attachment of dyes to antibodies in immunofluorescence.

Hydrogen bonding

- Force of attraction between a hydrogen atom in one molecule and a small atom of high electronegativity in another molecule.

- Can occur between dye and water it is dissolved in, hence are of little significance in aqueous staining.

- Hydrogen bonding is of significance in alcoholic dye solution

E.g.

  - Best's Carmine stain for glycogen; hydrogen bond formation between OH groups of the glycogen and H atoms of carmic acid

Solvent-solvent interactions

- A major contribution to stain-tissue affinity when using organic reagents or dyes in aqueous solution is the *hydrophobic effect*.

- The hydrophobic effect is the observed tendency of nonpolar substances to aggregate in aqueous solution and exclude water molecules.

- This phenomenon is utilized in staining lipids, which are hydrophobic. Hydrophobic stains will tend to dissolve into lipid rich regions of the section, highlighting them for analysis.

  E.g. Staining of fats by Sudan dyes.

Stain-stain interactions

- Dye molecules tend to attract each other, forming aggregates.
- Dyes which aggregate once bound to tissue → becoming resistant to removal from tissue spaces.
- Dye aggregation increases with concentration and ionic strength

  e.g. ↑ dye concentration/ionic strength→ Increased build up on tissue sections

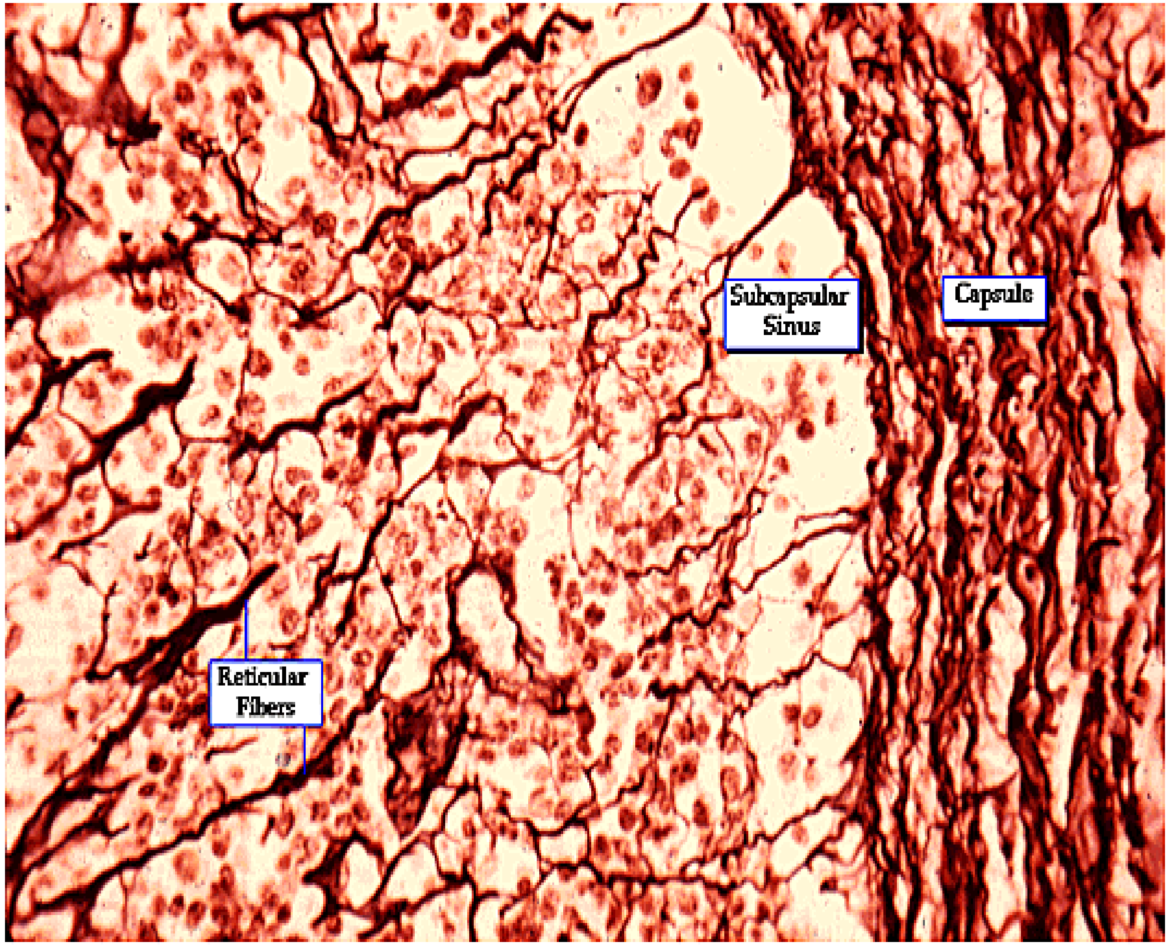
- E.g. With basic (cationic) dyes this occurs on substrates of high negative charge density, e.g. sulfated polysaccharides in mast cell granules by dyes such as toluidine blue.

Solubility

- E.g, when staining fat with Sudan dyes, an upper limit of staining intensity is set by the solubility of the dyes in the target substance, and is also influenced by the solubility in the staining bath solvent.
- Solubility is also involved in dye retention after staining.
- The stronger the reagent-reagent interactions, the lower the solubility.

Silver Impregnation (Silver staining)

- The mechanism is quite different to the effects of dyes.
- Tissue structures are actually plated with the silver rather than the silver being reversibly bound to the section.



Silver Impregnation (Silver staining)

Advantages	Disadvantages
They are stable and do not fade	Can be unreliable and capricious
Silver deposit is densely black	Silver solutions are often very alkaline
Very sensitive methods	Can give nonspecific background deposits (‘dirty preparations’)
Slender objects eg fine fibres such as reticulin or for slender bacteria such as spirochetes are thickened because they become silver-plated.	Tendency to stain everything they come into contact e.g. hands, laboratory coats, benches, glassware
	Some silver solutions have a tendency to become explosive if stored for more than 24h.
	Silver cannot be discarded into the drains as it is a heavy metal poison.
	Silver is expensive

Uses of silver

- Silver is easily reduced and any reduced silver acts as a catalyst for the reduction of more silver.

- Silver solutions are reduced during the impregnation, so silver techniques are primarily methods for reducing materials.

- There are three different ways of producing silver deposits.

i. Argentaffin reaction
ii. Argyrophil reaction
iii. Ion-exchange reactions

The Argyrophil Reaction

- An external reducer or developer is added
- In this case the developer does the main reduction and the tissue simply provides places where there are silver atoms to catalyse the reduction.

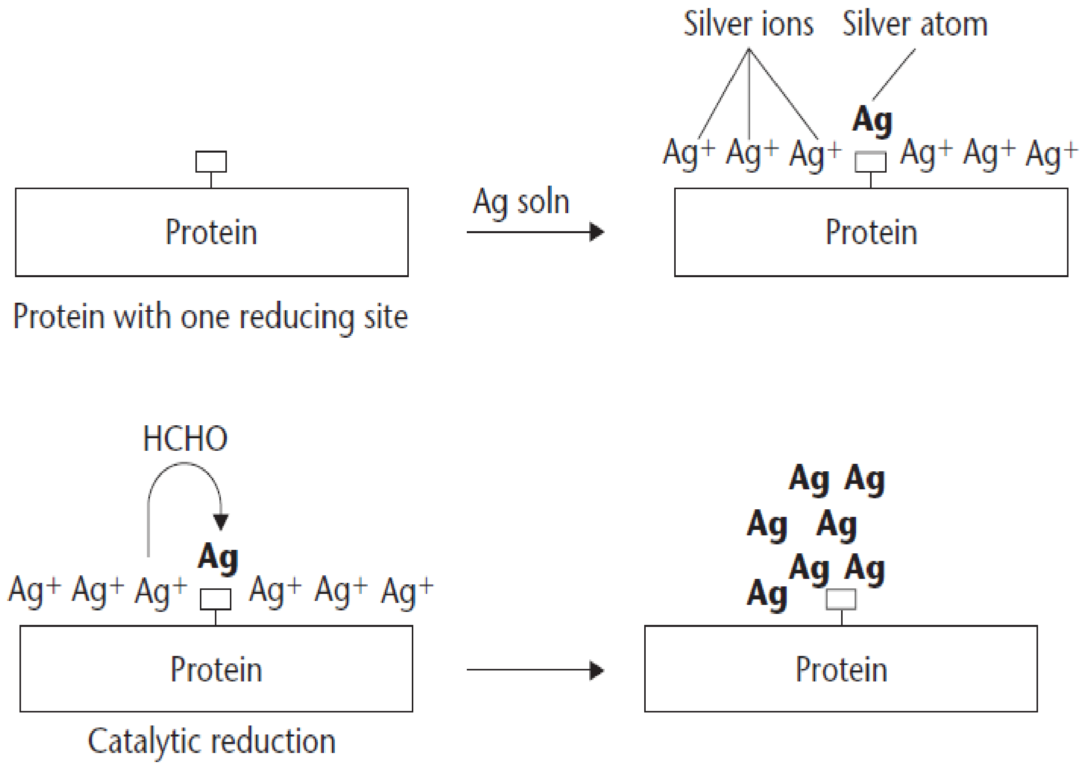


Figure 6.11
Silver deposition and reduction in the argyrophil reaction

The Argentaffin Reaction

- In this reaction, the tissue contains reducing groups (often aldehyde groups) that are sufficiently strong and present in sufficient quantity to give a visible deposit without added reducing agents.

- The reaction only needs the addition of the silver solution, such as in the Masson–Fontana technique.

Ion-exchange reactions

- Is used to detect mineralization of bone using the von Kossa technique.

- The section is treated with silver solution (silver nitrate) and the phosphates and carbonates in the mineralized bone form insoluble silver salts.

- The silver salts are then blackened by UV light or hydroquinone solutions.
    - $CaCO_3 + 2AgNO_3 \rightarrow Ag_2CO_3 + Ca(NO_3)_2$
    - Ag_2CO_3 (UV treated) $\rightarrow Ag_2O + CO_2$ Black

Automated Staining

Advantages	Disadvantages
Frees staff from a routine task that is relatively straightforward	Less flexibility
	Only feasible for techniques that are carried out for a large number of samples
	Does not lend itself to situations where different results are needed e.g. when photographing at low magnifications, an overstained section will give better results than the usual staining intensity.
	Demands reproducible reagents e.g. If there is a change in a reagent's staining properties, the machine will not recognize this and compensate for the change in the way that a person would
	Often cannot cope with large numbers of sections in a short space of time . But useful for absolute regularity with large numbers of sections needing the same treatment at the same time.
	Less flexible in producing single stains, even when they are already programmed for that stain

References

- Bancroft J.D. & Stevens A, (2013) **Theory & Practice of Histological Techniques**, 7th edition, Churchill Livingstone, NY, U.S.A.
- Cook, D.J (2006). **Biomedical Sciences Explained: Cellular Pathology**. Butterworth Heinemann, Oxford. ISBN: 10: 1948 42305
- Suvarna, S.K, Christopher L, Bancroft J.D, **Bancroft`s Theory & Practice of Histological Techniques**, 7th edition, Churchill Livingstone, NY, U.S.A.