Histochemical Techniques

Principles of staining:

During any histochemical application, we seek to stain some biological entity of interest. This whole process can be examined from the lens of following questions:

- Why any tissue component gets stained?
- Why stained tissue remains so at the end of the process?
- Why we do not see staining of all the components?

Before we move further, let us define stain.

The process of staining requires that biological entity of interest must be visually labeled by attaching or depositing a marker of characteristic color or form on or vicinity of the entity. The characteristic marker or the reagent used to generate the color is called as stain.

Interactions leading to staining:

For staining to happen the dye must be taken up by the tissue and it relies on dye-tissue or reagent-tissue affinity. By affinity, we mean the tendency of the stain to transfer from the solution onto the tissue or section. Greater the affinity, better is staining.

In thermodynamic terms, a system has a tendency to maximize its entropy so as to maximize its disorder. This also contributes to spontaneous staining when using appropriate reagent systems. This is explained by the fact that presence of dye in solvent and tissue system represents a more disordered system than dye in solvent only.

The affinity is mediated by the interactions between dye and tissue. These interactions are discussed below:

Interactions	Practical examples where the factor is important
Reagent-tissue interactions	
Coulombic attractions	Acid and basic dyes, and other ionic reagents, including inorganic salts
van der Waals' forces	Strongest with large molecules such as the elastic fiber stains, and final reaction products such as bisformazans in enzyme histochemistry
Hydrogen bonding	Staining of collagen by Sirius red, glycogen by carminic acid
Covalent bonding	Methods such as the Feulgen nuclear, PAS and mercury orange for thiols
Solvent-solvent interactions	
The hydrophobic effect	Staining systems using aqueous solutions of dyes or other organic reagents, e.g. enzyme substrates
Reagent-reagent interactions	Metachromatic staining with basic dyes, inorganic pigments in Gomori-type enzyme histochemistry, metallic microcrystals after silver impregnation

Table: Summary of all the interactions involved in dye-tissue interaction.

Coulombic attractions: They are also called as salt linkages or electrostatic interactions. They occur when dye and tissue structures have opposite charges and these charges are sufficiently strong.

E.g. Basic dyes and tissue components which are polyanions such as DNA or sulfated mucosubstances.

van der Waal's forces: These interactions are generally found to between all reagents and tissue substrates. But they become important when tissue or stain have extensively delocalized electronic systems. It is because such systems favor larger dipoles and greater polarizability.

Thus, proteins rich in tyrosine and tryptophan residues and nucleic acids having heterocyclic bases favor van der Waal's interactions. This is also true for dyes having large aromatic systems such as bisazo dyes, halogenated dyes such as rose Bengal.

E.g. van der Waal's forces are major reason for stain-tissue affinity when basic dyes such as Congo red and orcein are used for staining elastic fibers rich in desmosine and isodesmosine having polyaromatic acid structure.

Hydrogen bonding: It is not important for stain-tissue affinity if dye has been dissolved in aqueous solvent. This is because water molecules are much much greater in number than dye molecules.

But in certain cases, the substrate to be stained favors hydrogen bonding. This is seen during staining of collagen fibers and glycogen.

There is a related phenomenon called as halogen bonding and sometimes it contributes to the staining affinity. This is seen in staining by halogenated fluorescein dyes such as phloxine and eosin Y.

Covalent bonding: In this case, covalent bonds are formed between stain and tissue. There are several examples such as Feulgen nuclear stains and PAS reactive stains.

Sometimes, we see mordanting i.e. formation of polar covalent bonds between dye and tissue. The metal ions in dyes make a polar covalent bond with the substrates. E.g. staining by hematein dye.

Hydrophobic effect: This is major contributor to the stain-tissue affinity when organic reagents or dyes are used in aqueous solution. In this case there is no stain-tissue attractions and it relies on tendency of hydrophobic groups to cluster together in an aqueous environment.

E.g. Staining of fats using Sudan dyes which is prepared in a substantially aqueous solution.

Stain-stain interactions: These are also called as dye-dye interactions and are the major interaction seen in metachromatic staining.

Dyes can aggregate in aqueous solution due to hydrophobic effect and in case of aqueous and non-aqueous solutions both, van der Waal's interactions between planar dye molecules can lead to formation of the aggregates.

Dye aggregation increases as the dye concentration increases in the tissues.

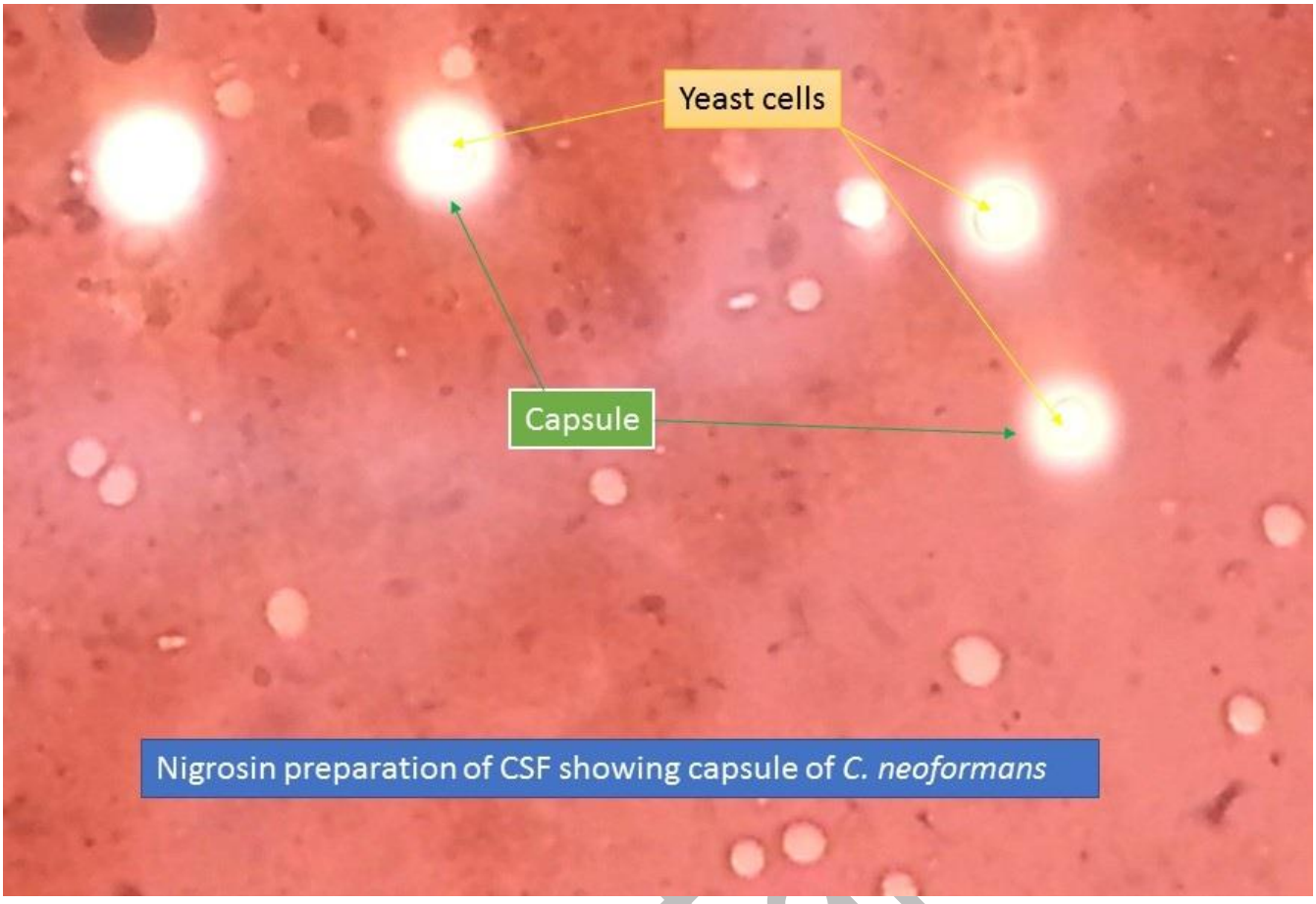
E.g. Toluidine blue, a cationic dye shows aggregation on substrates of high negative charge density such as sulfated polysaccharides in mast cell granules and gives rise to metachromatic staining.

Metachromasia is defined as staining of the tissue or its components such that color of the tissue-bound dye complex differs significantly from the color of the original dye complex leading to a marked contrast in color.

This happens because the spectral properties of the dye aggregates are very different from the monomeric dye. Thus, this form of staining is called as metachromatic staining.

Negative staining: This is applied when stain can't be taken up by the tissue targets. In this, shape of the structure is visualized by filling them or outlining them with a stain.

E.g. Demonstration of canaliculi in bone matrix using Schmorl's picro-thionine stain and microbial staining using nigrosine.



Retention of stain in the tissues:

Stain will be retained in the tissue after removal from the staining bath if:

- it has high affinity for tissue elements it is bound to.
- it has low affinity for solvents used during processing and mounting media.
- it dissolves in the processing fluid very slowly.

Ionic pigments such as Prussian blue which is generated in the Perl's method for iron, and lead sulfide produced in the Gomori-style enzyme histochemistry are insoluble in solvents used in histotechnology. This is also true for the microcrystals of metallic silver and gold produced by metal impregnation methods. Thus, their insoluble nature leads to stains' retention.

In case of other dyes such as azo dyes and substituted indigos the products of final reaction during staining are poorly soluble in water but can dissolve easily in hydrophobic medium such as alcohol or xylene. Therefore, in this case we use hydrophilic mounting media. The same logic is true for non-ionic dyes such as Sudan stains.

The routine basic dyes such as crystal violet or methylene blue are highly soluble in water and lower alcohols. Therefore, when sections are stained with these dyes they are dehydrated by passing them rapidly through alcohol gradient or using non-alcoholic solvents or by air drying. In case of acid dyes such as eosin Y and orange G and basic dyes with large aromatic systems such as Alcian blue we find poor solubility in alcohols.

The sections stained with the acid or basic dyes are mounted in non-aqueous medium as they can't extract the dye out of the section. As an alternative, the dyes can be immobilized and it is seen in many cases such as formation of iodine complexes in Gram's staining.

Stain selectivity

This process is important for staining the target and leaving an unstained background so that there is contrast and image is clearly visible so that observer can clearly identify or locate the entity of interest. This process is governed by certain factors which are discussed below:

Number and affinities of binding sites: This part helps us ensure that we have a stained target and unstained surrounding or differentially stained target and surroundings, so as to generate enough color contrast.

This can be understood by following examples:

Sudan dyes have high affinity for fat but low affinity for the surrounding proteins which are hydrated. Thus, they stain the fats only and outline their position.

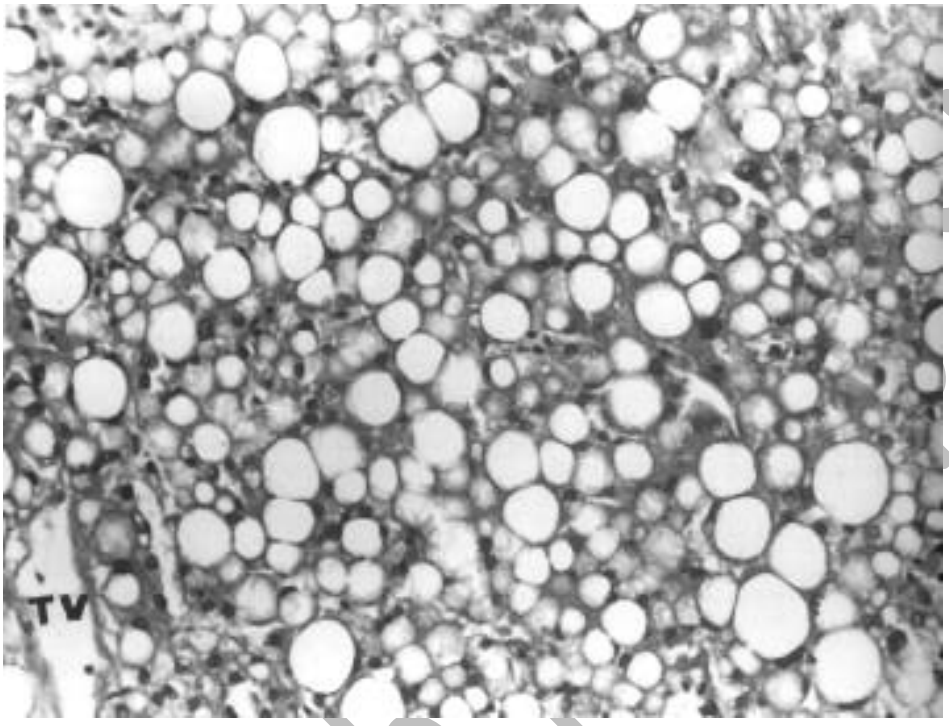


Figure: Tissue section stained with Sudan black dye.

Traditional staining using anionic-cationic pair of dyes such as Hematoxylin and eosin (H&E), Papanicolaou and Romanowsky stains are another example. Acid dyes are negatively charged and bind to the tissue parts having positive charge such as proteins in acidic conditions.

The tissue parts with negative structures are bound by the basic dyes as they are positively charged. It includes nucleic acids and sulfated glycosaminoglycans.

This leads to a two tone staining pattern where cytoplasm and nucleus are differently stained.

The affinity of the dye for the binding site is also affected by the solvent used to prepare the stain and its pH. Therefore, anionic or acid dyes are applied from acidic solutons, when proteins carry overall positive charge and basic or cationic dyes are applied from neutral or acidic solutions because under alkaline conditions proteins have an overall negative charge and can bind basic dyes.

Rate of reagent uptake

Can structures with equal stain-tissue affinities and equal binding site numbers be distinguished? This is possible if the rate of stain uptake, the rate of subsequent reaction, or the rate of loss of stain are not the same in the different structures.

Progressive staining may be rate controlled e.g. mucin staining using alcian blue or colloidal iron. Selectivity requires short periods of dyeing, during which only fast-staining mucins acquire color.

After prolonged staining, structures such as nuclei and RNA-rich cytoplasm also stain. Stains used in this way are often large and consequently slow diffusing,

Rate of reaction

Selective staining by reactive reagents may depend on differential *rates of reaction*. For instance, periodic acid can oxidize various substrates present in tissues. However, the histochemical PAS procedure uses short oxidation times, limiting coloration to fast-reacting 1,2-diol groupings of polysaccharides.

Rate of reagent loss

Differentiation or *regressive staining* involves selective losses of stain from tissues. Dyeing methods exploiting this include staining muscle striations with Heidenhain's iron-hematoxylin and myelin sheaths with luxol fast blue. In such procedures an initial non-selective staining is followed by solvent extraction, the dye first leaving permeable structures such as collagen fibers. By contrast, relatively impermeable structures e.g. the A and Z bands of muscle and myelin sheaths, retain stain longest.

Rate control of reagent loss is also important in silver staining of nerve fibers. During impregnation, silver cations bind non-selectively to many tissue sites. Subsequently, the sections are treated with developer which reduces silver cations to silver metal. The rate of this reduction reaction is critical: if too fast because of high concentration or high reactivity of the developer, silver grains are deposited non-selectively throughout the tissue.

Whereas if reduction is too slow, no staining occurs because most silver ions diffuse away into the solvent before they are reduced. Selective staining occurs when silver ions diffuse from the background but are retained in less permeable entities e.g. nerve fibers, nucleoli and red blood cells where they are then reduced.

Metachromatic staining

Even when neither affinity nor rate controls staining patterns, selective coloration remains possible. For instance, basic dyes such as methylene blue and toluidine blue are absorbed by a variety of basophilic tissue substrates. Chromatin stains "orthochromatically" blue, but cartilage matrix, mast cell granules and mucins stain "metachromatically" reddish purple. This metachromasia is due to dye aggregate formation in the porous, polyanion rich sites.