Dyes and Stains: from molecular structure to histological application

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1. ABSTRACT

In the present review, the chemistry of dyes as well as the interaction mechanisms between tissue and dye has been detailed, and also some of the key factors affecting the selectivity of dyes by certain cellular structures have been mentioned. Moreover, due to the relevance that histological stains have acquired in biomedical research, some of the most common stains have been described, pointing out previous and current applications in basic and applied research.

2. INTRODUCTION

Histology is basically the study of microscopic structures of cells and tissues, a discipline that has acquired importance since the development of the microscope in the 1500-1600, which allowed seeing for the first time certain structures with more detail. However, as most fixing materials have a refractive index similar to the tissue, it lacks of contrast making its observation difficult. In order to overcome this issue histological stains were developed, being nowadays widely utilized to study cell structures. Specific intracellular and extracellular elements are visualized at microscopic level by using dyes able to react with defined tissue components.

Staining is basically the artificial coloration of a substance to facilitate its examination by the use of a colored organic molecule called dye. The process can be technically performed progressively or regressively. In the first case, the dye only interacts with the tissue until the proper stain is reached, while in the latter, the tissue is overstained and the excess of dye is afterwards removed (Figure 1). On the other hand, with the purpose of identifying more than one cellular component in a sample; the staining can be achieved by either simultaneous or successive addition of dyes, which selectively stain certain structures. Thus, double, triple and also multiple staining can be performed.

Histological staining, unlike to immunohistochemistry, does not use antibodies to identify specific molecules and therefore lacks of the inherent specificity of an antigen-antibody interaction (1). However, despite its limitations, histological staining has been extensively used in pathology and research, not only because it is easy to perform, but also because of its relatively low economic cost. It also allows identifying several types of cell and provides invaluable information about cell structures, tissue morphology and architectural changes associated with certain diseases.

Although the first staining dyes were tried in the 17th century and some of them have been used for long time (2), it is hard to establish a uniform theory of tissue staining because binding mechanisms between dyes and cell components are quite heterogeneous. For this reason, conventional histological procedures should be analyzed in close relation with the dye chemistry and the morphological behavior of cell structures.

The aim of the present review is to provide necessary information for professionals that develop across different disciplines. On the one hand, it gives useful details to further understand the chemical basis of tissue-dye interactions, constituting a significant tool for the synthesis and design of new dyes. At the same time, to deepen the knowledge of the chemical interactions involved is relevant for the development of possible new histological techniques, in order to satisfy researcher's needs. On the other hand, the review provides important information about methodological details and recent applications of stains, in order to help those who are just getting into the histology or need an upgrade on histological procedures to implement those techniques.

3. BASIC PRINCIPLES OF DYE STAINING MECHANISMS

3.1. Dye chemistry

Dyes are colored organic compounds able to selectively bind to tissue components. Although many dyes originally have poor affinity by tissues and weak capacity to produce color, these characteristics can be easily improved by chemical modifications.

A chromophore is a part of the molecule capable of absorbing strongly light at certain wavelength and transmitting or reflecting at others. Examples of chromophores are C=C, C=O, C=N, N=N, N=O, NO_2. Essentially, these groups isolated absorb light in the UV region; however, if they are part of an extended conjugated pi system the substance suffers a bathochromic shift to the Visible region and becomes colored (Figure 2A). An important chromophore used in dyes is the quinoid arrangement (Figure 2B). However, a compound can be colored but still not stain because it lacks the ability to bind to tissues. To turn a colored compound into a useful dye in histology, incorporation of an ionizable group that binds to tissue components is required. The ionizable group can be classified in acids, such as –COOH, -OH, $-SO_3H$, and basic, that include primary, secondary and tertiary amines ($-NH_2$, -NHR, $-NR_2$) (3). Usually, these functional groups are called auxochromes. They are attached to the chromophore modifying the ability of the chromophore to absorb light, altering the wavelength or the absorption intensity. In this sense, phenol is a white power; introduction of three nitro groups led to a yellow solid, the well-known nitro dye picric acid (Figure 2C).

Despite these structural requirements, some compounds are unable to bind to a tissue by itself. To overcome this issue, further chemical manipulations in combination with metal salt formation, commonly refer as mordant, are used. Hematoxylin is the most important mordanted dye (see section 4.1).

Because in the past the dyes were primarily produced for industrial purposes such as textile dyeing, a wide variety of names were given to them, sometimes naming the same compound in different ways depending on the manufactures, and usually without considering the chemical structure. When the dyes acquired importance in biological sciences, it was necessary to overcome the confusions and the Color Index was established by the Society of Dyers and Colorist (3). It is a standard list that considers all dyes, their synonyms and their chemical structures, being the chromophoric group the main feature considered to do the classification. Because it is an extended list, some of the most important histological dyes are mentioned below and some of their chemical structures are shown in Figure 3:

a) Nitro dyes, with the nitro group $–NO_2$ as the chromophore, such as picric acid (Figure 2C).

b) Azo dyes, such as Congo red and Sudan IV, with the azo group –N=N- as the chromophore attached to two aromatic rings.

c) Antraquinone dyes, such as Alizarin, with a quinone structure as the chromophore, located in the middle of a three fused aromatic ring system.

d) Xanthene dyes, including rhodamines, pyronins, acridines, phenolphthalein and fluoroscein derivatives, characterized by a quinoid structure in a three fused aromatic ring system

e) Quinone-imine dyes that include azins, oxazines, thiazins and indamins. It is a more heterogeneous group that shows a quinoid structure in a three fused aromatic ring system with a heteroatom such as nitrogen, oxygen and sulfur, depending on the dye.

f) Phenyl-Methane dyes that include diphenyl-methanes, diamine triphenyl methanes, triamino triphenyl methanes among others. They have three aromatic rings, being one of them in a quinoid structure, and also contain different numbers of amino groups.

3.2. General interaction mechanisms

The interaction mechanisms between tissues and dyes rely on the same forces that occur in other organic compounds, and they can be classified in physical and chemical.

3.2.1. Physical interactions

In physical stains the dye may be dissolved in the stained substance, absorbed on the surface of the tissue structure or precipitated within the tissue. The processes are favored by environmental factor as pH, ionic strength, temperature, etc. Most of fat stains work on this preferential solubility principle (2).

3.2.2. Chemical interactions

Unlike physical interactions, chemical interactions are more common in histology and imply a tissue-dye binding. Figure 4 summarizes the major classes of interactions showing the characteristic strength.

Ionic bond or electrostatic interaction is the most important in histological staining and involves electrostatic attractions between opposite charges of dye and tissue. Thus, an atom with extra electrons interact with another that is electron deficient and the overall neutrality is established (4). Because proteins normally contain both acidic and basic amino acids, this type of bond depends on the ionization state of tissue components, being pH sensitive. In acidic solutions, the high hydrogen concentration associated to the low pH favors ionization of amino groups, yielding positively charged proteins able to bind to negatively charged dyes. In the opposite side, the absence of hydrogen ions associated to alkaline solutions allows formation of carboxylic or sulphate anions, able to bind to positively charged dyes. Salt concentration is another important factor that affects ionic bonds. In this sense, at high salt concentration a competition between the dye and the salt is produced, inhibiting the staining. On the contrary, if salt concentration is low, more dye than salt is present in the solution and the staining is enhanced.

Another type is the covalent binding, although it is only important in a few stains. This type of bond is the product of a reaction between the dye and the tissue and often a new C-C bond is formed. Covalent bonds are very strong and cannot be broken by procedures usually encountered in histological staining. An example is the Schiff's reagent that covalently attaches to aldehydes in carbohydrate identification by Periodic Acid- Schiff staining (PAS) (see section 4.2.1.1).

Hydrogen bonds are produced between hydrogen and a small highly electronegative atom, usually oxygen or nitrogen, being necessary that both involved groups be close enough. Chemical groups that qualify as potential hydrogen binding site include primary and secondary aliphatic amines, secondary aromatic amines, amides, hydroxyls and carbonyls among others (4). This type of bond is less preponderant in histology than ionic bonds.

Van der Waals are not classical bonds and include all remaining bonding mechanisms that involve dipoles (5). They are short-range forces between any two atoms, without specificity, although a small distance between both atoms involved is necessary together with a match in the shape of tissue component and dye. This type of force probably does not play a relevant role in the binding process although it could be important in the tissue-dye selectivity process.

The last type of forces are the hydrophobic interactions also only important in the selectivity, which maintain the dye in the tissue by means the exclusion of water from hydrophobic regions, stabilizing the two groups involved.

3.3. Factors involved in tissue-dye interaction

Staining is basically a two steps process. The initial step is ion exchange, which increases the dye ion concentration around tissue constituents, and the next step is the actual binding between dye and the corresponding groups in tissue components (6).

In the overall staining, the fixation procedure and the dye concentration are important factors (6); however, several relevant physicochemical processes are involved in the selective uptake of the dye by a biological specimen. The relevance of net or partial electric charges (Z) involved in the different types of binding and also the importance of pH and salt concentration in dye-tissue interactions were previously introduced. In addition, other factors such the overall molecular weight (MW), the size of the aromatic/conjugated pi system (CBN) and the hydro- or lipophilicity dominated the selectivity of dye-tissue interaction

3.3.1. Dye selectivity based on the electric charge (Z). histological classification

Because two dyes with similar chemical structure may have quite different uses in histology, another classification based on its histological application is more useful. The most widely known classification is based on the electric charge and includes: basic dyes, acid dyes, neutral dyes, amphoteric dyes and non-ionic compounds (7).

Basic dyes are characterized by having a positive charge and therefore are also known as cationic dyes, being Hematoxylin one of the most popular. This type of dyes can react with anionic groups as carboxylates, phosphates and sulphates, and because they can bind to phosphate groups of nucleic acids (DNA and RNA) are regularly used as nuclear stains. Thus, anionic components that attract basic dyes are called basophilic. In contrast, acid dyes possess a negative charge and they are also called anionic, being the Eosin the most relevant example. Acid dyes can react with cationic groups, mainly present in proteins and other cytoplasmic components, and therefore are widely used as cytoplasmic stains. In this context, cationic structures that attract acid dyes are commonly called acidophilic (6).

Neutral dyes are constituted by an acid and a basic dye able to produce staining. It does not imply that solution of these dyes has a pH 7, but merely that both cationic and anionic structures can be colored. Although many acid and basic dyes are able to form neutral dyes, only a few combinations are used being the Romanowsky stain the most common procedure (6). Other types of dyes are the amphoterics, which also have both cationic and anionic groups but both are present in the same molecule. Although both charges are present, these dyes can only act as a basic or acid dye being the pH a crucial factor to determine the behavior. As it is known, each molecule has a specific isoelectric point (IP) that corresponds to the pH at which positive and negative charges are canceled each other, and the overall charge on the molecule is zero (8). Thus, at pH below the IP the positive charges are favored and the component is considered as a cationic dye, while at pH above the IP the negative charges are favored being the compound considered as an anionic dye.

The last types of compounds included in this classification are the non-ionic dyes. Although unable to produce a heterolytic dissociation and therefore unable to chemically interact with the tissue, they are included here because they have color and are able to stain certain tissues by dissolving in them.

3.3.2. Influence of MW, lipophilicity and CNB in dye selectivity

The size of the dye, given by its molecular weight, is one critical factor involved in dye binding, being able to directly modify the dye diffusion rate (4). In this sense, larger dyes diffuse through a tissue section much slower than small dyes, being this property widely used in some staining procedures. Moreover, an increase of temperature can increase the rate of staining by increasing the diffusion of the dye molecule (9).

No less important is dye lipophilicity, which determines its ability to interact with tissue components. In this context, Dapson RW (4) described a hydrophobic index useful to characterize several dyes, although the logarithm of the octanol-water partition coefficient (log P) has been more extensively used to determine the lipophilicity of dyes. This coefficient is defined as the ratio of concentrations of a compound in a mixture of two immiscible liquids at equilibrium, precisely water and octanol (10). Thus, negative values of log P are associated with hydrophilic dyes while for all fat staining the log P is above 0.

Another relevant parameter to consider is the conjugated bonds number (CBN), determined from the structural formula by counting bonds in conjugated pi systems (4). Although generally acidic dyes stain proteins strongly in acid dye baths, and basic dyes do better in basic dye baths, there are some exceptions such as the staining of amyloid by Congo red, in which case the high CBN= 43 of the dye acquires relevance. The binding between Congo red and amyloid depends on non-polar attractive forces, being these strong interactions due to the large conjugated pi system present in Congo red (Figure 5).

Therefore, all these parameters are known as bonding parameters and are useful tools to understand tissue-dyes interactions selectivity, assisting to predict which dyes will be successful to stain certain substrates, and also being valuable for new dyes design (11). Figure 5 shows some of the discussed parameters for certain dyes indicating for example that Acid Fuchsine is twice the size of Azure B, suggesting that the latter is able to diffuse faster. It is also possible to see that Acid Fuchsine is highly hydrophilic while Oil red O is a lipophilic dye, being one of the most used dyes to stain fat.

4. MAIN HISTOLOGICAL STAINS: MECHANISM AND APPLICATIONS

Hematoxylin-Eosin (H and E) is certainly the most widely used staining to identify nucleus and cytoplasm respectively in cells. Because of the versatility of Hematoxylin, several types of this dye are used in histology, although Mayer's, Harris's and Gill's Hematoxylin are the most popular used in H and E.

As previously introduced, Hematoxylin is not really a dye because it is unable to stain (12), nevertheless it can be converted into an active dye called Hematein by an oxidation process known as ripening (Figure 6A). In the past, the oxidation was naturally produced by the exposure of Hematoxylin to air and sunlight, but because it requires long time to occur, a chemical oxidation is currently performed (2). In this sense, various agents can be used as oxidants, including sodium iodate in Mayer's Hematoxylin, mercuric oxide in Harris's Hematoxylin and also potassium permanganate in others Hematoxylins (13).

Even after the oxidation, the direct staining with Hematoxylin is still unsuccessful being necessary the presence of a mordant. It is a metal salt able to act as a link between the dye and the tissue by a not completely understood mechanism, although one likely interaction is a dative covalency. The combination between a mordant and a dye is known as "lake", and the resulting complex is often positively charged, acting as a cationic dye at low pH. In this context, the main salts used as mordant contain iron, chromium, tungsten or aluminum, although the latter is the most common in H and E (14). Aluminum ammonium sulfate salt is found in Mayer's and Harris formulations, and aluminum sulfate salt in Gill formulations (5). While the presence of a Hematein-Al^{3+} complex in these salts is widely accepted, the details of this structure have been subject to discussion for long time (15). As it was early described, Hematoxylin by itself is not the active dye, and although the correct name of the dye would be Hemalum or alum Hematoxylin, the staining solution is traditionally referred to as Hematoxylin.

In addition to that, various acids are usually added to Hematoxylin solutions in order to decrease the pH, which reduces the non-specific background by increasing the selectivity of the stain for nuclei. Some of the more used are citric acid in Mayer's Hematoxylin and acetic acid in Gill type Hematoxylins.

All alum Hematoxylin can be used as either progressive or regressive stains (16). In a progressive stain, the Hematoxylin is left to interact with the tissue until it reaches the proper stain, being the chromatin primarily colored. By this procedure, it may be necessary to try different incubation times with Hematoxylin on the optimization stage. On the contrary, in a regressive stain, the Hematoxylin overstains chromatin and cytoplasm, requiring an additional step to pull out the excess of color (17). This step, known as differentiation, is commonly performed using a weak acid, usually an alcoholic acid due to Hematoxylin high solubility in alcohol. Thus, the acid competes with the dye for binding to anionic sites removing the Hematoxylin from some places, while the structures which retain the dye more effectively remain colored. It is important to note that in progressive stains milder forms of Hematoxylin are used to stain the nucleus, such as Gill's and Mayer's Hematoxylin, while stronger Hematoxylins such as Harris formulations are used in regressive stains.

After the stain endpoint has been reached by either progressive or regressive methods, the nucleus is initially stained with a purple color. Because it does not provide a good contrast with the usual red counterstains, it is necessary to change the purple color into a deep blue color, a step known as bluing (5). To achieve this, sections are immersed in a solution with pH between 5 and 11, being regular tap water commonly used due since it is alkaline enough compared to the Hematoxylin. Eventually, if pH of tap water is not appropriate, it is possible to use a weak ammonia solution or Scott's tap water substitute (TWS), an aqueous bluing solution of pH 8 (17).

Hematoxylin is one of the most representative basic dyes, able to stain acidic structures including the nucleus due to its DNA content, as well as organelles containing RNA such as ribosomes and rough endoplasmic reticulum. However, a large number of theories have been proposed in order to explain the staining mechanism of nuclei. Some observations indicate that positive charges of the complex Hematein-Al^{3+} are attracted to negatively charged sites of the tissue, mainly to phosphate residues of nucleic acids, with the involvement of electrostatic forces. Nevertheless, it has also been proposed that these forces only mediate the initial attraction followed by their replacement by more stable forces as covalent or coordinate bonds (15, 18). In this context, the pH of the staining solution is of great importance, being necessary a pH higher than the isoelectric point of nucleic acids in order to promote the presence of negative charges in phosphate groups, being them more easily attracted by Hematoxylin (19).It has also been proposed that Hematoxylin could bind to strongly basic nuclear proteins as the histones associated with nucleic acids (20). In this sense, some studies have shown a weak staining of nuclei after nuclear DNA digestion, suggesting that the attachment of Hematoxylin to chromatin does not require DNA (21). By contrast, other evidence has shown that DNA digestion prevents the nuclear staining suggesting that DNA is essential for the dye-tissue binding.

Eosin is the routine dye used as a counterstain in H and E stain, and it is formed by the reaction between Bromine and Fluorescein (22). There are two Eosin variants used in histology: Eosin Yellowish also known as Eosin Y, and Eosin Bluish also known as Eosin B, being the former one the most popular (Figure 6B). Eosin Y also can be classified based on the dye solubility, being possible to find aqueous and alcoholic Eosin Y. In addition, a color enhancement can be achieved by adding Phloxine B to the Eosin (5).

Conversely to Hematoxylin, Eosin is an acidic dye able to bind to ionized cationic groups of proteins such as β-amino groups of the side-chain of lysine and guanidine groups of arginine (21). Because almost all proteins contain these two amino acids, Eosin can bind nearly all the structures present in a tissue. However, there are some exceptions unable to take the Eosin, such as glycogen and proteoglycans of extracellular matrix which are negatively charged, in addition to granules of mast cells. Although the staining mechanism by Eosin is not fully understood, due to the chemical nature of the dye it is possible it binds to the tissue by ionic forces, although Van der Waals forces cannot be ruled out. In this context, the pH is also important, being the Eosin usually utilized in a slightly acid solution to promote the protonation of amino groups of proteins, which are more easily attracted to the negatively charged Eosin (23).

Other anionic dyes different from Eosin can also be used as counterstains, but it is important to avoid those with strong stain power such as Methyl blue, because they may possibly cover the nuclear staining (24).

Hematoxylin and Eosin staining is routinely used in histology and histopathology laboratories because it provides a very detailed view of the tissue. In clinical laboratories, tissue samples are almost always initially processed for H and E which allows identifying several cell structures including the cytoplasm, nucleus and some extracellular components. Then, based on the information obtained by H and E it is decided if other special stains are necessary.

Despite the improvements in the pathology diagnose based in the current advances in molecular pathology and cell biology, the information obtained by H and E is of great importance and it is still invaluable. In an overall view it is possible to determine the ratio nucleus/cytoplasm and identify cell organization or disorganization that can be helpful in the diagnostic of some diseases. In a more detailed view it is also possible to see alterations in nuclear size and shape, as well as identify changes

in chromatin patterns that can be typically associated with some types of cancers (25). Moreover, Hematoxylin followed by counterstaining with Orange G is one of the most popular stains used in vaginal smears to look for signs of malignancy (26).

4.2. Special stains

4.2.1. Basic cellular components identification

4.2.1.1. Carbohydrates

Carbohydrates are a large group of substances found both in normal tissues and under pathological conditions. They can be basically classified in three main groups: glycans, glycosaminoglycans and glycoproteins. Glycans, also known as polysaccharides, are polymers of monosaccharide units, being the glycogen the most common example. Glycosaminoglycans are linear polymers composed by repeating disaccharide units, which form proteoglycans if they are connected to matrix proteins. Glycoproteins are composed by one or multiple oligosaccharides covalently attached to a protein, and can be found on the outside surfaces of cells, in specific organelles as Golgi and also in secreted products as mucus (8).

Because both the absence and the increase of any of these substances as well as changes in their distribution pattern can be a pathological findings, it is of great importance to identify these cellular components (2). In the present review two of the most known stains for carbohydrate are exposed.

One way to identify carbohydrates is through the staining of their ionized acidic groups by using cationic dyes, like Alcian blue (Figure 7). Despite several cellular components are capable to be negatively charged, such as phosphates of nucleic acids and carboxylate groups of glycoconjugates and proteins, by selecting a certain pH only some of them are selectively ionized. Thus, working at pH 1.0 only sulphated glycosaminoglycans and glycoproteins are stained with Alcian blue, while at pH 1.7-3.2 N-acetyl glycoproteins are well stained (5). It is important to mention that even at low pH, Alcian blue can be selectively used to stain carbohydrates without stain nucleic acids because the size of this cationic dye is large enough to avoid its penetration and association with DNA and RNA (5).

Working with cationic dyes it is of particular importance the concept of metachromasia. Metachromasia is the characteristic of a cell or tissue component of staining in a color different from the ordinary color of a dye (orthocromasia), and it is related to the ability of some tissues structures to aggregate dye particles and create a new color. This phenomenon is characteristic of some basic dyes of thiazine group, especially thionine, azure B and toluidine blue. These blue dyes stain metachromatically to red or purple color. The use of such dyes to identify charged mucins and proteoglycans is one of the oldest histochemical techniques for carbohydrates (2).

One of the major uses of Alcian blue lies in its ability to identify goblet cells, glandular specialized cells found in some tissues able to secrete mucus. Because goblet cells are not found in normal stomach, it is traditionally accepted that goblet cell intestinal metaplasia is the leading candidate for the origin of gastric cancer (27). Thus, the loss of parietal cells in gastric mucosa and their replacement for aberrant presence of goblet cells identified by Alcian blue are of great importance in the diagnoses of gastric cancer (28). Alcian blue is also used to study Barrett's esophagus (BE), an acquired condition in which the squamous epithelium of distal esophagus is replaced by intestinal epithelium with presence of goblet cells (29).The relevance of this disease lies in the fact that BE is a major risk factor for the development of esophageal adenocarcinoma (30). It is also common to see the combination of Alcian blue with other stains to achieve specific objectives, like High Iron Diamine-Alcian blue to identify neutral mucins, sialomucins and sulphomucins in biopsies with intestinal metaplasia (31).

The other approach to identify carbohydrates is the Periodic Acid-Schiff (PAS) staining, one of the most useful histological stains. This method is based on the presence of an adjacent pair of hydroxyl groups in sugars, which can be oxidized with periodic acid yielding two aldehyde groups. In the next step these groups are detected with the chromogenic Schiff's reagent, which covalently combines with the aldehydes to produce a red-purple compound (2). PAS strongly stains glycogen and certain types of mucins, and it is also useful to identify basement membranes due to the sugar moieties of its proteoglycans.

One important control utilized in PAS stain is the inclusion of a glycogen digestion step before the actual staining, typically performed with alpha-amylase although it is more known as diastase digestion. It is known that the hydrolysis of glycosidic bonds of glycogen is produced by the enzyme, leading to glycogen breakdown in disaccharide water-soluble molecules, which are then removed from the tissue section in a washing step. The result is the glycogen removal before PAS staining (5). The whole procedure involves two duplicate slides subjected to PAS procedure, with and without previous digestion. Thus, a positive reaction in sections without digestion together with the loss of staining in sections with digestion confirms the existence of glycogen.

Glycogen is normally found in liver, endocervix and muscle; however, it can be pathologically present in carcinomas of liver, kidney, pancreas and bladder among others, in seminomas and mesothelioma and also in the glycogen storage disease (32). PAS staining has also been suitable in the study of alveolar soft-part sarcoma, a very rare malignant tumor characterized by the presence of intracytoplasmatic PAS-positive diastase-resistant crystals (33). It is also currently helpful to differentiate alveolar soft-part sarcoma from rhabdomyomas, since the latter presents PAS-positive intracytoplasmic granules that disappear if the pre-treatment with diastase is performed (34). Moreover, PAS has been useful for many years to study the α1-antitrypsin deficiency,

a disease able to produce liver injury due to the retention of a mutant α1-antitrypsin in the endoplasmic reticulum of hepatic cells. By using PAS it is possible to identify PAS-positive diastase-resistant intrahepatic globules that represent dilated endoplasmic reticulum with the aggregated mutant protein (35-36). In addition, PAS staining has great importance in the study of renal morphology, providing information about structural changes associated to kidney damage. Thus, thickening of the glomerular basement membrane and the expansion of the mesangial matrix are changes typically found in diabetic nephropathy and easily visible by PAS (37-39).

Even nowadays, with the development of new and more sophisticated techniques, PAS staining is still widely used in histology, and moreover it has been recently combined with immunofluorescence in order to perform a quantitative glycogen analysis in tissue sections (40).

4.2.1.2. Lipids

The term lipid involves a wide range of compounds whose common characteristic is their insolubility in water, being soluble in chloroform or other organic solvents. Lipids include fatty acids, glycerides, cholesterol esters, phospholipids, phosphoglycerides and sphingolipids among others, which possess different biological functions. Some of them are the main storage source of energy, others the major structural elements of biological membrane and others play key roles as enzyme cofactors, chaperones, hormones or intracellular messengers (8).

The main inconvenient to identify lipids is their inability to remain in tissue sections through the routine procedure, being easily removed with the solvents used during tissue preparation. In order to avoid this issue it is necessary rapidly freeze the sample instead of embedding it in wax or paraffin, and then cut it in a refrigerated microtome called cryostat.

The most common way to stain lipids is with Sudan dyes, which include a variety of dyes such as Sudan black, Sudan III, Sudan IV and also Oil red O. Lipids staining do not involves chemical bonds dye-tissue, but the stain depends on physical principles based on the preferential partitioning of the dye in the lipid compartment rather than in the solvent. However there are some evidences supporting the idea that the staining may be also an adsorption process related to the temperature, dye concentration and physical state of the fat (5).

Sudan III was one of the first dyes used, being later replaced by Sudan IV because it produces a stronger staining, however nowadays both have been largely replaced by Oil red O due to the deeper color that it produces. Oil red O is widely used to identify the most hydrophobic lipids, while Sudan black is able to stain these lipids in addition to other less hydrophobic such as phospholipids and sphingomyelins. The main problem of using Sudan black is its inefficacy to stain crystalline cholesterol; however, this drawback can be solved if bromine pre-treatment is included in the staining procedure. Bayliss OB *et al* (41) not only demonstrated a stronger staining of crystalline cholesterol by Sudan with preliminary bromination, but also the reaction of free fatty acids and lecithin were markedly enhanced in that condition. They also showed that lipid staining with Oil red O was generally improved after bromine treatment, although cholesterol crystals were only moderately stained. By using bromine, crystalline cholesterol is converted to derivatives that are oily at room temperature, thus permitting the uptake of the dye. Thus, almost all lipids are stained with this method.

The principal application of Sudan dyes is the identification of lipid accumulation, being currently utilized to study adipogenesis in several tissues like liver and brown adipose tissue as well as in different cell lines (42-45). Although the staining is important in normal conditions, it is mainly useful for the understanding of several pathological situations. Cardiovascular diseases are one of the main clinical conditions that affect the population, being Sudan IV and Oil red O great tools to study these pathologies because their capability to demonstrate atherosclerotic lesions. Lipid stains are also useful to evaluate the effect of diets, clinical drugs and herbal medicine in the evolution of atherosclerotic lesions (46-47). Others works have reported Sudan dyes utilization to study a wide spectrum of clinical conditions. For example, in brain autopsy of patients with autosomal dominant leukodystrophy, Itoh K *et al* (48) reported presence of sudanophilic macrophages in white matter. In addition, Piva E *et al* (49) described lipid droplets in muscle biopsy and in neutrophils of blood smears of a patient with neutral lipid storage disease.

Because of the great importance of lipid identification, it has been developed a combination of Oil red O staining with immunofluorescence suitable to study lipid droplets together with proteins, allowing further knowledge about subcellular localization of proteins and lipids (50). It is also important to mention that there are other more sophisticated to detect specific lipid groups such as osmium tetroxide alpha naphthylamine for cholesterol esters, and gold hydroxamate for phosphoglycerides, which are more frequently used to study lipid storage disorders (2).

4.2.1.3. Nucleic acids

As is largely known there are two nucleic acids called deoxyribonucleic acid or DNA and ribonucleic acid or RNA, constituted by nucleotides which are composed by a sugar, phosphoric acid and a nitrogenous base. Both nucleic acids are involved in the preservation, replication and expression of the hereditary information (8).

The first described reaction to identify DNA was Feulgen stain. In this method, aldehyde groups produced by acid hydrolysis of DNA with HCl react with Schiff's reagent resulting in a purple staining. Because RNA is not hydrolyzed by HCl treatment, this technique is proper to selective DNA identification (2).

The use of Feulgen staining dates many years ago. An investigation about DNA content of some tumor cells was described in 1956 by Atkin NB *et al* (51), who also compared the quantitative data obtained with information from clinical, pathological and cytological studies, trying to establish some relation. Moreover, Feulgen staining has been used in the past to demonstrate the banded karyotype of mouse, in which each chromosome is identified by the relative Feulgen stain density in specific regions and by a characteristic pattern of alternating light and dark bands (52). In addition to that, several authors have reported the utilization of Feulgen to detect nuclear and cytogenetic abnormalities such as chromatin condensation, karyorrhexis, presence of vacuoles, altered number of micronucleus and changes in nuclear size and shape (53-55).

Due to the relevance of nucleic acid identification in tumor pathology, DNA analysis has become an important tool for cancer diagnostic. However, precise measurement only can be achieved if the dye amount bound per nucleus is proportional to the DNA content, being Feulgen reaction accepted as the most appropriate and precise stoichiometric procedure for DNA staining (56). In this context, in the last years and due to the existence of more evolved techniques, the determination of DNA content by Feulgen in combination with image analysis, microspectrophotometry and flow cytometry has allowed to assess different degrees of ploidy in physiological and pathological conditions (57-59).

Another interesting staining to identify nucleic acids is the combination Methyl green-Pyronin Y. Although both dyes are able to stain both nucleic acids, it is known that working at certain pH the Methyl green is specific for DNA, staining green the nuclei, while Pyronin Y is specific for RNA staining red the nucleoli. For this reason, it has become a technique of invaluable relevance to pathologists (2). Due to the limited availability of Methyl green, it has been replaced by Ethyl green, and several studies have been performed in order to compare both green dyes, confirming the reliable and reproducible DNA staining with both dyes (60-61).

Methyl green-Pyronin Y has been used with different purposes. For example, it has been utilized to evaluate cytomorphometric changes in the nucleus and nucleoli in squamous cell carcinoma of different grades. This analysis showed to be useful to identify grades of cell differentiation and thus facilitate the early detection of premalignant lesions (62). On the other hand, Amaral FR *et al* (63) (2012) have reported the utilization of this stain to assess the apoptotic rates of some types of tumors, being able to design an apoptotic index useful to compare those tumors.

Finally, it is important to mention that currently there are several more complex and developed techniques to study DNA and RNA as well as nuclear abnormalities. The aim of the present review is only to describe the histological tools for the study of nucleic acids, recognizing its limitations.

4.2.2. Connective tissue identification

Structurally, cells and extracellular matrix (ECM) are the connective tissue constituents. Within the cells, fibroblasts are the most common type, although macrophages, mast cells, plasma cells and leukocytes are also present; each of them responsible for different functions. The ECM, the major component, basically consists of ground substance and fibers. The ground substance is an amorphous complex of anionic macromolecules, proteoglycans and glycoproteins with fairly high water content, while connective tissue fibers are composed of structural proteins, being collagen fibers, reticular fibers and elastic fibers the three most important. Collagen is the most abundant protein in the body, being possible to find more than 20 different types of it, while reticular fibers are known to mainly consist of collagen type III and are present as networks of thin and highly glycosylated fibers. Elastic fibers are formed by the protein elastin and, as the name suggest, they constitute a highly elastic network (64). Although many functions can be attributed to connective tissue, to be responsible for providing structural support for tissues and organs of the body is the most prominent. In addition to that, connective tissue gives metabolic support to cells, acting as the medium for nutrients and waste products diffusion.

Because the ECM is the major connective tissue component and because different type of fibers can be altered in several conditions, the main staining techniques to identify fibers are described in the present review.

Trichrome stainings are the most widely used to visualize collagen fibers and one of the most utilized stain in pathology. These techniques utilize two or more acid dyes and a polyacid to differentiate two basic components in contrasting colors, commonly to demonstrate collagen fibers in contrast to smooth muscle (7). Amino groups of tissues are able to ionically bind to the acid dyes, and because two acid dyes are used it would be expected that both dyes stain the same component. However, by controlling the ionized state of dyes and tissue and applying the mass action principle, it does not happen. Thus, the staining is invariable performed at low pH, usually by adding acetic acid, because as it was previously mentioned it favors amino group ionization, yielding positively charged proteins that bind to acid dyes. Moreover, because the staining is usually performed in a sequential way, called multi-step, the control of the staining is facilitated.

The first step is the nuclear staining, commonly performed with Hematoxylin. However, because the low pH can remove the alum Hematoxylin it is replaced by ferric hematoxylins, such as Weigert's Hematoxylin, which resist removal by acids (5). Then, the first acid dye is applied, known as the plasma stain, usually red and of intermediate molecular weight. It is applied the necessary time to stain all the tissue, even erythrocytes, muscle and collagen. In the next step the polyacid is added, commonly molybdophosphoric or tungstophosphoric acids. These are high molecular weight molecules, colorless and with an overall negative charge, whose function is to displace the plasma stain from collagen (5).When enough stain has been displaced the polyacid is removed, followed by the addition of the second acid dye called the fiber stain, usually blue or green. It is applied the necessary time to displace the polyacid and deeply stain the collagen, without replacing the red staining of other components.

It is important to mention that the selective staining of erythrocytes is also possible, although it is an optional step not always performed. If it is desired, an acid dye of low molecular weight, such as Picric acid or Martius yellow is applied before the plasma stain. Thus, the small dye enters through the erythrocyte membrane and it is trapped within the cell (5).

To summarize, the sequence in a multi-step trichrome staining is the following:

1. Nuclear stain
2. Erythrocytes stain (optional)
3. Plasma stain with first acid dye
4. Displacement with polyacid
5. Fiber stain with second acid dye

Masson's and Mallory's Trichrome are the most common multi-step trichrome stainings, which basically differ in the plasma and fiber dyes used, although the nuclear dye can also be different. In both cases Bouin's picric-acid is the recommended fixative solution, but formalin-fixed sections can be used although a re-fixation for 1 hour at 56ºC or overnight at room temperature is suggested before performing the staining (5).

As opposed to multi-step methods, there are one-step methods, being Gomori's Trichrome currently the most popular. The distinctive characteristic of these techniques is that all dyes are combined in a single solution. By adding all the reagents at the same time, various factors need to be strictly controlled to obtain a satisfactory stain. Thus, while multi-step procedures are frequently more tolerant to minor changes, after any modification in a one-step method a re-standardization of the whole procedure is required.

Although these techniques were developed many years ago, they are still largely used in pathology. Masson's Trichrome is the preferred tool to identify cardiac fibrosis in various heart dysfunctions, and it is also utilized to study the protective effect of some therapies as well as the efficacy of several treatments on cardiac morphology (65-68). Pulmonary fibrosis and the inflammatory response associated are also of great relevance nowadays, being Masson's Trichrome useful to evaluate collagen deposit in lung. In addition to that, the possible development of new therapeutic drugs and the effect of known drugs on pulmonary fibrosis have also been studied by using Masson's Trichrome (69-71). Chronic kidney disease and other nephropathies are tightly associated with renal fibrosis and represent actual relevant diseases. In this context, Masson's Trichrome has been widely used in several studies focused in elucidating the intracellular mechanisms associated with these conditions (72-74). Another current application of Masson's Trichrome includes the study of muscular dystrophy, a condition characterized by the replacement of muscle with fibrotic tissue, being collagen identification especially suited to assess the degree of disease progression (75). Moreover, the stain is useful to differentiate denatured collagen and viable collagen in the dermis of burn wound, and thus to evaluate the lesion evolution (76). The stain, sometimes combined with other tools, is of great help to distinguish between myofibroma and smooth muscle lesions, a procedure reported as difficult to be achieved only with immunohistochemistry (77). It is important to highlight that the current relevance of Masson's staining is also due to the development of new informatics tools, which have made possible to quantify the staining of a color by image analysis, in this case blue color (78-79).

Mallory's and Gomori's Trichrome, although less used than Masson, also have been utilized to identify collagen and muscle fibers in clinical studies and also in research studies (80-82). But without doubts, one of the main past and current applications of Gomori stain is the study of mitochondrial cytopathies associated to several clinical conditions (83-86). This syndrome groups a heterogeneous number of diseases, all of them characterized by the presence of red staining materials in the subsarcolemmal and intermyofibrillar regions of irregularly shaped fibers, known as ragged red fibers, corresponding to an accumulation of diseased mitochondria which are essentially identified by a modified Gomori's Trichrome stain in skeletal muscle biopsies (87).

Elastic fibers are other important ECM components that can be identified by histological staining. Although numerous techniques have been described, Verhoeff-Van Gieson and Weigert's resorcin-Fuchsin are the most popular, although Orcein staining is also used.

The first step in both Verhoeff and Weigert's stains involves rupture of disulfide bridges, which normally keep the elastic fibers highly cross-linked. Thus, the oxidative treatment with iodine in Verhoeff stain and with permanganate in Weigert's stain is able to convert the disulfide bridges into anionic sulfonic acid derivatives. The obtained compounds are highly basophilic and able to react with the corresponding cationic dyes, Hematoxylin in Verhoeff stain and Basic Fuchsin in Weigert's stain (5). Both reactions are enhanced by working with a high electrolyte concentration solution, which avoids the dye uptake by chromatin and RNA. Because both are regressive stains, tissue sections are overstained being necessary a posterior differentiation step. In Verhoeff it is accomplished by using excess of ferric acid which attracts and displaces the dye from some tissue elements, while elastic tissue retains the dye longer due to the stronger affinity given by dye-protein hydrogen bonds. In Weigert's stain this step is performed with acid alcohol (5). The final step is the counterstain, usually carried out with Van Gieson or iron Hematoxylin.

The main limitation of Verhoeff stain resides in the inability to satisfactorily stain both coarse and fine elastic fibers in a single section. Usually, if fine fibers are optimally stained the others are under-differentiated, and if thicker fibers are optimally stained the finest fibers can be decolorized. Despite this issue, the staining is largely utilized by optimizing the differentiation for the fiber of interest. Verhoeff stain is currently used in clinical pathology to characterize several diseases. Wick MR *et al* (88) have studied the interstitial tissue in asbestosis disease, showing the presence of a fibroelastotic process more than the traditional fibrotic process described. On the other hand, the study of granuloma annulare pathogenesis, a type of benign skin lesion, has shown damaged elastic tissue and collagen, while an important loss of elastic fibers was seen in dermatochalasis, a disorder characterized by lax eyelid skin (89-90).

Weigert's resorcin-Fuchsin staining is also extensively applied in the present, for example to investigate the remodelling process in idiopathic interstitial pneumonia, which shows a progressive vascular fibroelastosis in lung (91-92). In addition to clinical studies, Weigert's stain has also been utilized in basic research to evaluate the ventricular myocardium lesion and the repairing process after experimental induced hypertension, showing the increase of elastic fibers as a key event in post-infarct repair (93).

Unlike the techniques recently described, Orcein is a progressive stain for elastic fibers and for this reason does not require a differentiation step, being simpler and easier to perform (5). Even the staining solution is simple to prepare, being many times the chosen method due to technical inexperience. Orcein staining has been used to identify elastic fibers in many and varied conditions such as nonalcoholic steatohepatitis, temporomandibular joint displacement, skin lesions by UV light, atheromatous lesions and also in aging processes (94-98).

Many years ago Shikata's modified Orcein technique was described. In this case, prior to orcein addition, peroxidation of tissue sections is performed with acidified permanganate followed by oxalic acid decolorization (99). This stain not only demonstrates elastic fibers but also, and most important, is able to demonstrate the hepatitis B surface antigen (HbsAg). Thus, Shikata's modified stain is useful to identify the classical ground glass hepatocytes often found in chronic hepatitis B and characterized by a granular cytoplasm, being this stain an important tool for routine screening of HbsAg (100). Moreover, because Shikata's modified method is also able to demonstrate the copper associated protein (CAP), it has been utilized for the diagnosis of specific childhood cirrhosis, advanced liver disease and copper associated liver disease (101-102).

The other ECM components are the reticular fibers, and because they are extremely thin, around 0.5-2.0 µm, they cannot be visualized by regular staining being necessary a silver impregnation. Although silver is not a dye, it is commonly included with dyes because it is useful to visualize specific tissue structures, thereby being regularly used in histology (2). There are several types of silver stains for reticulin, such as Wilder and Gordon and Sweet among others, which have some differences but in all cases the staining principle is based on the formation of colloidal metallic silver. Because after exposure to silver solution an external reducing agent needs to be applied to complete the reaction, it is conventionally called an argyrophilic reaction, unlike to an argentaffin reaction in which the reducing agent is present in the tissue (103).

The first step in any reticulin staining regardless the chosen method is the oxidation, which converts sugars hydroxyl groups of the fibers to aldehydes, and that can be performed by phosphomolybdic acid or potassium permanganate. Then, because reticulin fibers have little affinity for silver a sensitization step is required, a procedure usually done by treatment with heavy metal salt solutions such as ferric ammonium sulfate. Thereafter the silver impregnation is accomplished with silver nitrate or silver diamine, and the sensitized sites accept the silver. However, because reticular fibers are unable to reduce the silver, tissue sections are still uncolored being necessary to add the reducing agent formaldehyde (5). At this point, the formaldehyde is oxidized to formic acid while the silver complex is reduced to metallic silver visible as brown-black. Subsequently and as a way to improve the contrast, silver deposits can be transformed into purple-black color by treatment with gold chloride, followed by the removal of unreacted silver by adding sodium thiosulfate. The last step is the counterstain, usually performed with nuclear fast red (5).

Reticulin visualization is useful to outline the architecture of parenchymal organs such as liver and spleen, but the utilization of silver stain has also been reported to study benign and malignant bone marrow disorders associated with pathological increase in reticular fibers (104-105).

4.2.3. Minerals identification

Several minerals are daily required for various metabolic functions in the body, being calcium one of the most important. Calcium has a fundamental role in bone mineralization, but it is also important for other body functions like muscle and blood vessels contraction, nerve conduction, clots formation as well as for regulation of some enzymes activity. This nutrient is normally present in bones and teeth as hydroxyapatite, but abnormal deposition can also be found, being associated with lung or lymph nodes infections, necrotic tissue in lesion of atherosclerosis, some type of tumors, and nephrocalcinosis among others. Currently two main stains are used to identify calcium: Von Kossa and Alizarin red S.

In the body, calcium deposits are mainly found as calcium phosphate and calcium carbonate. Thus, although Von Kossa staining does not identify the metal by itself, it is largely used because the anionic portion of the metal salt can be demonstrated. Because other carbonate or phosphate salts can also be visualized, the reaction is not completely specific for calcium (5). However, because the body usually contains more calcium salts than other salts; a positive reaction is tightly associated with calcium presence, although it is important to take into account that metal salts are in fact stained. Moreover, Von Kossa is unable to demonstrate calcium oxalate.

Von Kossa technique is based in a metal substitution reaction by using silver nitrate. Thus, positively charged calcium is displaced by positive silver which binds to the negative portion of the salt, creating silver phosphate or silver carbonate, and then colored to brown-black in the presence of strong light. Later, the unreduced silver is removed followed by a counterstain step. In order to increase the reliability of the staining and to ensure that black material is calcium, treatment of a duplicate section with 0.5% aqueous hydrochloric acid for a few minutes has been suggested. In this way calcium is removed and a negative reaction should be observed in the section (5). It is important to mention that fixative solutions containing strong acids as picric acid should be avoided in the tissue processing because calcium deposits may be removed by the acid.

Because the ability to identify calcium salts, Von Kossa staining is even nowadays widely used to study mineralization processes in several tissues and conditions, being the identification of articular cartilage calcifications one of the most common uses, for example to deep the knowledge of osteoarthritis (106). The biological characteristics of alveolar osteoblasts in patient of different ages have been also evaluated with this stain by identifying variations in number and size of calcium nodules (107). Heart diseases and calcifications in circulatory system are currently of great relevance, being Von Kossa staining a useful tool to identify calcifications in arteries and aortic valves, as well as to study pharmacological treatments and the potential protective effect of new drugs (108-109). Others recently described applications of Von Kossa staining include nephrocalcinosis identification and determination of osteogenic differentiation of periosteal cells by visualizing of calcium nodules, being also interesting the possibility to characterize the molecular pathways associated to this process (110-111).

Alizarin red S, unlike Von Kossa, is more useful when small amounts of calcium are present because it is more sensitive. It is a chelating stain in which the anionic dye binds to calcium, although it is also able to bind to other metals like manganese and barium (20). However, because high concentrations of these metals in the body are unusual, a positive staining is tightly associated with calcium deposit.

The staining procedure for Alizarin technique is quite simple, including a first incubation step with Alizarin red solution, followed by removal of excess dye and a final quick dehydration in acetone instead of the typical alcoholic dehydration (5). Thus, a birefringent bright orange-red precipitate is formed. The only important consideration to take into account is the pH of the staining solution, which can vary in different protocols. Thus, working at pH 4.8-7.2 calcium was stained orange-red but other structures were stained faintly pink, and moderated diffusion was observed. On the contrary, working at pH 9 calcium deposits were stained red while all other structures were unstained, with background staining prevention due to the inability of impurities to react at alkaline pH (20).

The most common applications of Alizarin red staining are similar to those of Von Kossa, such as the recognition of calcification in coronary arteries and aorta, conditions strictly associated with increases in morbidity and mortality in end stage renal patients (112-113).Moreover, the staining is also helpful for the development of possible therapeutic drugs able to suppress mineralization deposit in the vascular system (114). As many others stains, Alizarin red is not only useful for tissue sections but it can also be applied to plated cells, acquiring great importance in cell biology. Thus, in the field of osteoporosis, osteoblast mineralization has been widely described by using Alizarin red S, being this stain also useful to demonstrate the effect of drugs and hormones on bone metabolism regulation. Furthermore, utilization of Alizarin red S has also facilitated the understanding of signal transduction pathways involved in bone metabolism (115-117).

Copper, although is present as traces in the body, is another essential metal required for example by the cytochrome oxidase enzyme and others oxidoreductase enzymes (8). In this context, because copper is present in very low amounts, any increase can be highly toxic and dangerous for the body, acquiring importance its identification. Several methods have been described to demonstrate copper, however none of them is completely specific for the metal, being possible to stain others metals as well. Rubeanic acid is one of the suitable reagents for copper demonstration, although p-Dimethylaminobenzydine rhodanine, also known as DMABR, is generally preferred because is a more sensitive staining (118). The latter is a chelating agent able to bind to the copper associated to proteins, and for this reason it has been suggested that others proteins-metal complexes can be

nonspecifically stained. The procedure basically includes the incubation of the sample with DMABR, excess dye removal and counterstain in Hematoxylin, giving as a result a red-orange copper precipitate.

Histological applications of these stains are limited because of the rare conditions associated with copper overload, being Wilson's disease the most characterized, followed by less common disorders like Indian childhood cirrhosis (119). Clinical features of Wilson's disease are due to a failure in tissue copper excretion and decreased copper incorporation into ceruloplasmin (120). In this context, both Rubeanic acid and DMABR have been useful for pathologists for many years in order to identify copper in biopsies of patient with different conditions associated to deposition of this metal (119, 121). However, the precise correlation between histological findings and copper amount in different stages of copper overload has been discussed for long time. In this sense, a good correlation has been reported in initial stages, although the stains fail to demonstrate subsequent increases in metal depositions (122-123). Thus, in addition to microscopic visualization of copper in tissue sections, it has been recommended quantify absolute copper levels in tissue in order to obtain a more precise diagnostic of Wilson's disease (121).

4.2.4. Pigments identification

Iron is an essential metal for several biological processes, however highly increased deposits may have clinical relevance. In the organism, iron is mainly stored in liver by binding with ferritin, a protein able to hold up to 4500 Fe^{3+} atoms, keeping the metal in a soluble and non-toxic form that can be rapidly utilized if iron needs are increased (124). However, if iron tissue levels are even higher it is stored as hemosiderin, a more stable form of iron storage. Thus, the metal deposited in this way becomes less available to supply iron needs (124). Hemosiderin is often found in storage organs as liver and in tissues involved in hemoglobin phagocytosis and degradation, thus contributing to iron recycling processes (2). For this reason, hemosiderin is without doubt one of the most important endogenous pigments for pathologist.

Although a trained eye can visualize Hemosiderin as a golden-brown refringent precipitate in H and E staining, Perl's staining also known as Prussian blue is the specific histochemical reaction traditionally used to identify this pigment. In the first step, the ferric iron bound to proteins is released by denaturing the binding with hydrochloric acid, forming ferric chloride. Then, the iron is combined with potassium ferricyanide to produce ferric ferricyanide, an insoluble blue compound known as Prussian blue. The last step is the counterstain, usually performed with a red dye like neutral red (2). It is important to mention that the relevance of this stain lies in the specificity to react with ferric iron, excluding the visualization of ferrous iron (5).

One of the most common diseases associated with iron overload is the Hereditary Hemochromatosis, an autosomal recessive inherited disease with high incidence in Northern-European populations. The most common form of Hemochromatosis is due to a mutation in HFE gene; however, mutations of one of at least other three different iron-metabolism genes (TfR2, HJV or HAMP) can lead to this metabolic abnormality. This pathology is characterized by increased intestinal iron absorption and excessive metal deposition in tissues, mainly liver (125). In this context, Perl's staining is an important tool to study and semi-quantify iron deposits, not only in liver but also in other affected organs of patients with this clinical condition (126-127). However, iron overload can also be secondary to a wide variety of disease such as chronic hepatitis C infections, cirrhosis and aceruloplasminemia, being Perl's staining also the chosen stain to visualize the metal (128-130). In addition, because iron is necessary to cell proliferation, it has been strongly associated with various types of malignant tumors, being hemosiderin identification also relevant to characterize the tumors and determine the significance of iron overload on cancers progression (131-132).

Prussian blue is also of great importance in biological research, mainly in the study of iron metabolism, a field that has had significant progress in the last decades. In this context, iron detection in key organs has been of great relevance to further understand the regulatory mechanisms involved in iron imbalance (133-135).

Another endogenous pigment of the body is melanin, primarily found under normal conditions in the skin and in the substantia nigra in the brain, although its presence can also be pathologic (2). The two most common methods used to identify melanin are Masson-Fontana silver and Schmorl's ferric ferricyanide.

Masson-Fontana reaction is based on the fact that melanin is argentaffin, being it able to reduce silver. Thus, the first step is the tissue treatment with an ammoniacal silver solution where the reducing groups of the tissue convert silver ions into colloidal metallic silver, colored brown-black. Next, color intensification can be produced with a gold chloride solution, which produces silver replacement by gold, followed by the counterstain step usually performed with neutral red or nuclear fast red (5). It is important to mention that this method is not completely specific for melanin because it is also able to demonstrate substances with similar reducing properties such as argentaffin cell granules or lipofuschins.

Unlike other largely used staining, Masson-Fontana is not a routine technique and it is more focused on the specific detection of melanin in malignant melanomas as well as in some benign tumors derived from melanocytes (136). Furthermore, many years ago it was reported the atypical use of Fontana-Masson as a relatively specific stain for the diagnostic of infectious caused by certain Cryptococcus, a procedure that is also currently applied (137-138).

Schmorl's staining is the other technique to identify melanin pigments, although less common and even less frequently utilized. It is based on the treatment of tissue sections with a solution of ferric chloride and potassium ferricyanide. Thus, ferric iron is reduced by melanin to ferrous iron, which is then combined with ferricyanide to produce ferrous ferricyanide, a blue pigment (5).

5. SUMMARY AND PERSPECTIVES

The significant development of the science in the last decades increased the need and the interest to know cellular and structural details, and it was possible due to the microscopic identification of cells and their components, being improved even more with the development of histological techniques. A histological staining is basically the procedure of staining some cellular structures by using dyes, which besides providing color must be capable of binding to certain tissue components but not to all of them.

In this review, the main features of staining mechanisms and the relevant properties of dyes for its use in histology were outlined. In addition, some of the applications performed in the last decades were described. Because there are many histological techniques for the detection of several tissue components and it would be impossible to do a complete revision, the attention was centered in the most commonly used stains. A summary of the main application are showed in Figure 8.

Although most of the stains were developed many years ago they are still in use, being some of their advantages the low economic cost required to perform a stain and the high availability of the necessary reagents in almost every laboratory. In addition to that, stains are usually easy to perform being of great importance a good reproducibility of the technique.

In the last decades, the need to evaluate pathological lesions and disease progression through histology has led to the need to estimate the amount of certain stains in a relatively accurate way. At the beginning it was achieved by semi-quantitative scoring techniques that are usually performed by more than one operator to achieve a more reliable result. Then, more sophisticated and reproducible tools were developed thanks to computer advances, and nowadays the use of digital imaging for recording microscopic findings together with the availability of software for image analysis have improved the assessment of histological stains.

On the other hand, among the disadvantages of histological stains, the lack of the inherent specificity of an antigen-antibody reaction is the main limitation, impeding the identification of a specific protein. However, it has been described that histological stains have certain features necessary to specifically recognize cellular components such as collagen, minerals, nucleus and even DNA. In addition to that, in the last years several combinations of histological stains with fluorescence, immunohistochemistry and also electronic microscopy have been described, pointing out the versatility of histology and the relevance of its utilization.

Taking into account advantages and disadvantages, histological stains continue to be a unique tool, due to its simplicity and reproducibility providing valuable information about cell structures and tissue morphology and pathology, thus contributing to several research fields.

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Figure legends

Figure 1. Schematic representation of staining procedure: A) Progressive stain, B) Regressive stain.

Figure 2. A) Extension of conjugation and bathochromic shift. B) Aromatic benzoid and quinoid structures. C) Picric acid retrosynthetic pathway from phenol.

Figure 3. Some typical dyes classified by their main structural features showed in color.

Figure 4. Types of dye-tissue interaction.

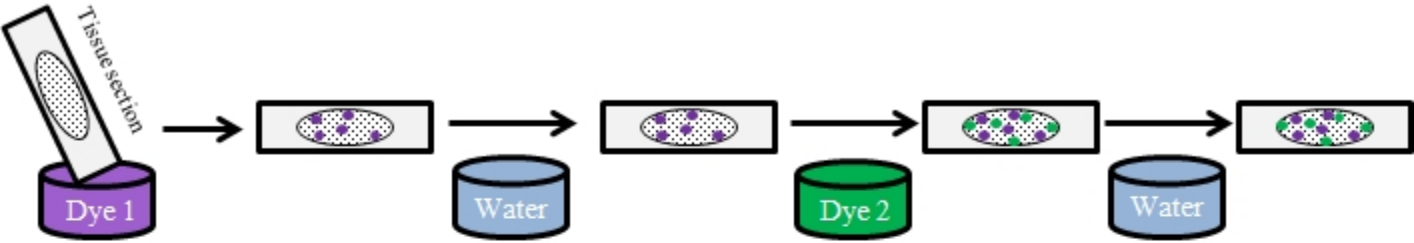
Figure 5. Examples of some dyes and their corresponding bonding parameters.

Figure 6. A) Oxidation of Hematoxylin, B) Chemical structure of Eosin.

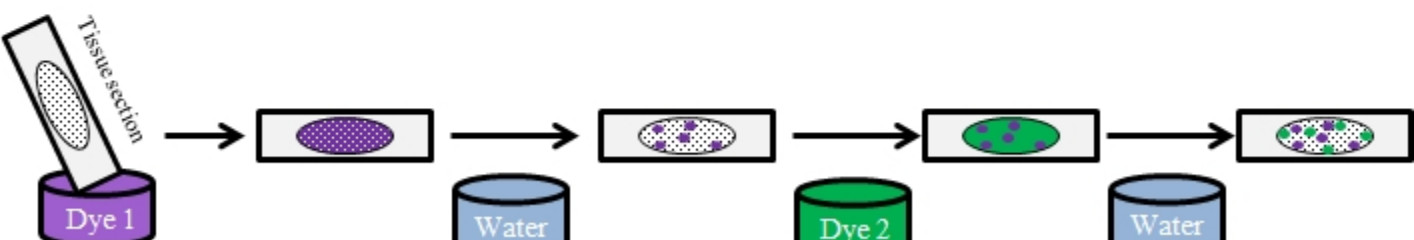
Figure 7. Alcian blue structure.

Figure 8. Summary of the main histological stains.

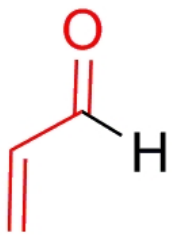
Running title: Dyes and stains in histology

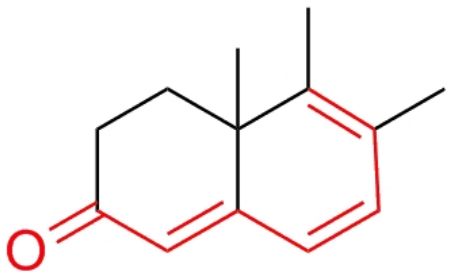

Tissue section
Dye 1
Water
Dye 2
Water

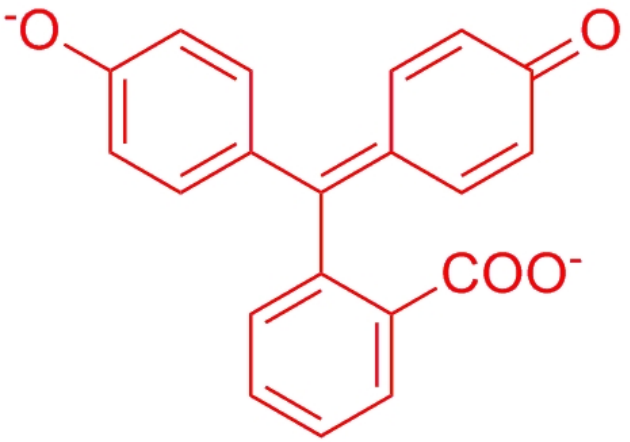
A


Tissue section
Dye 1
Water
Dye 2
Water

B





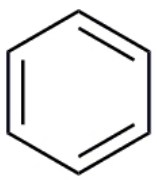


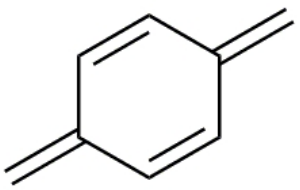
λ_{max} 207 nm
no color

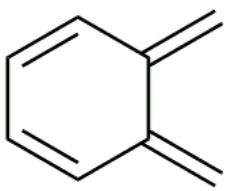
λ_{max} 388 nm

λ_{max} 552 nm
red

A





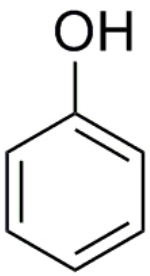


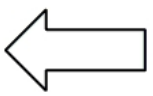
benzoid

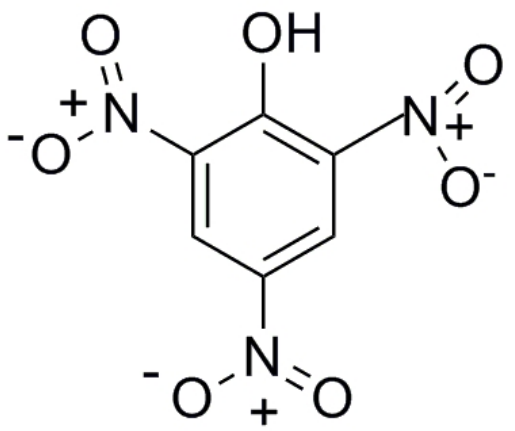
p quinoid

o quinoid

B





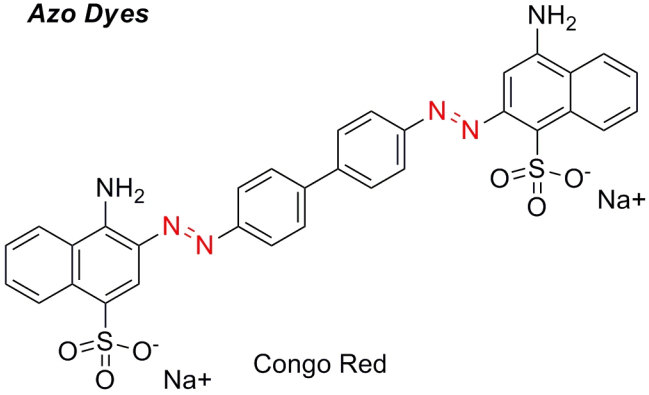


phenol
white

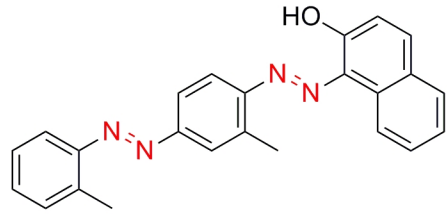
picric acid
yellow

C

Azo Dyes

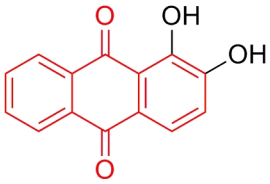


Congo Red



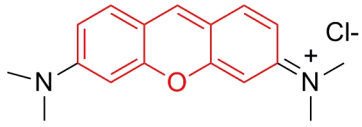
Sudan IV

Antraquinone Dyes

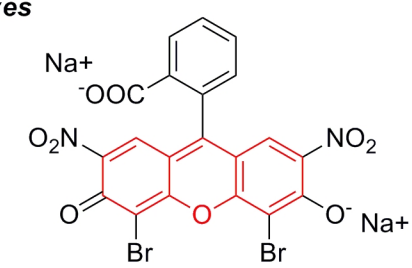


Alizarin

Xanthene Dyes

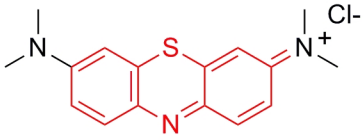


Pyronin Y



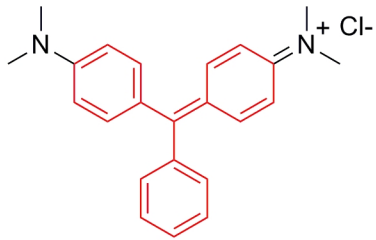
Eosin B

Quinone-imine Dyes

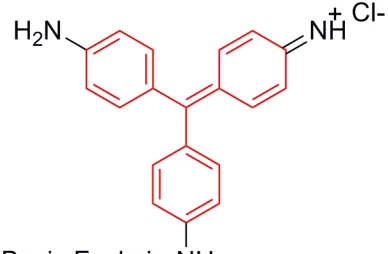


Methylene Blue

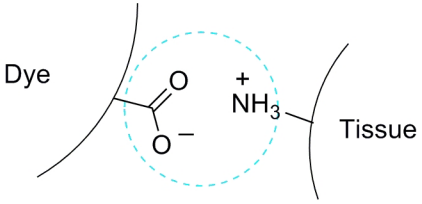
Phenyl-Methane Dyes



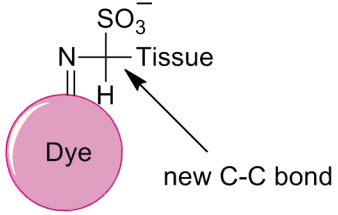
Malachite Green



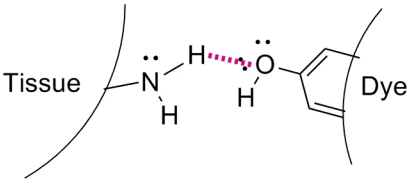
Basic Fuchsin NH_2



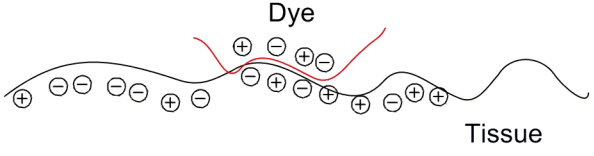
electrostatic attraction
(40-110 kcal/mol)



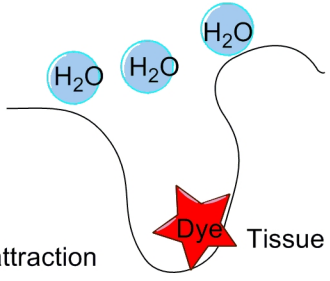
covalent bond
(35-212 kcal/mol)



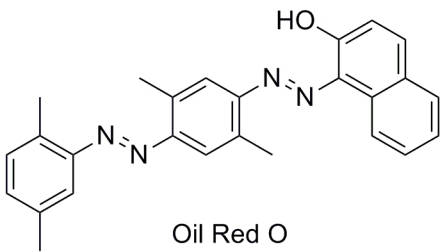
hydrogen bond
(2-7 kcal/mol)



van der Waals attraction
(1-2 kcal/mol)

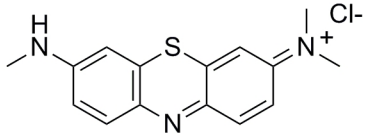


hydrophobic attraction
(4-8 kcal/mol)



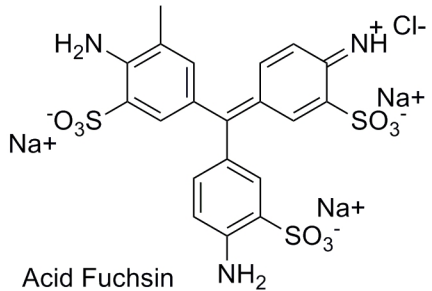
Oil Red O

Z: 0
MW: 408
Log P: 9.4
CBN: 30



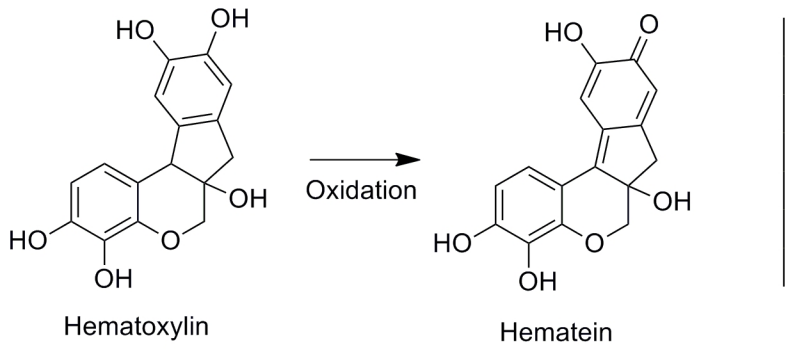
Azure B

Z: +1
MW: 270
Log P: -0.7
CBN: 18



Acid Fuchsin

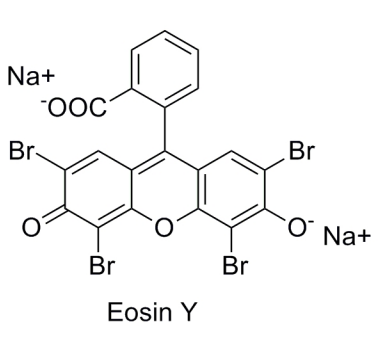
Z: -2
MW: 538
Log P: -11.5
CBN: 24



Hematoxylin

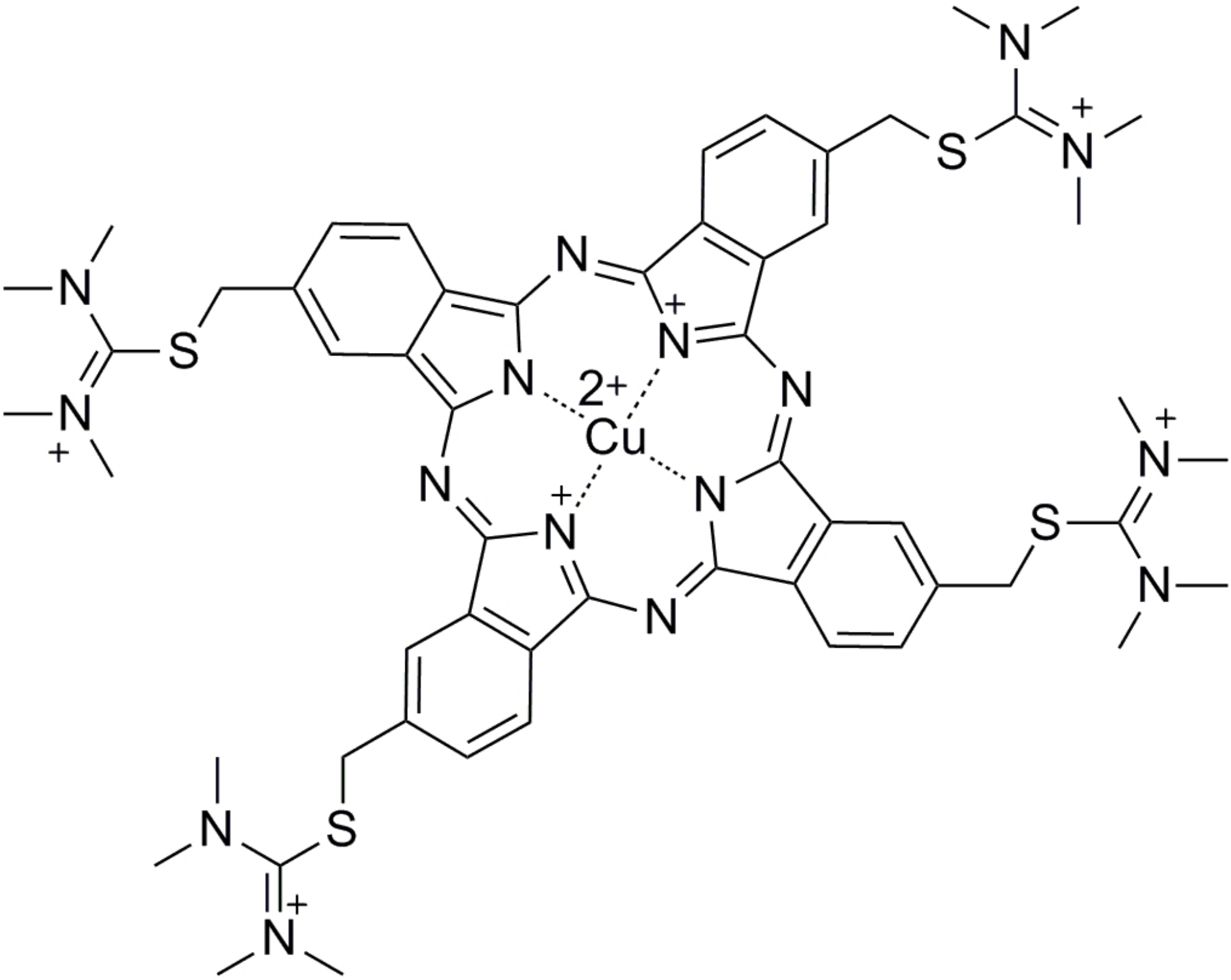
Hematein

A



Eosin Y

B


N
S
N
+
N
+
N
N
N
2+
Cu
N
+
N
N
N
N
S
N
+
N
S
N
+
N
+
N
+
N
S
N
+
N

Carbohydrates (*section 4.2.1.1)*
Alcian Blue
Periodic Acid-Schiff (PAS)

Lipids (*section 4.2.1.2*)
Sudan III, IV
Sudan Black
Oil red O

Nucleic acids (*section 4.2.1.3*)
Feulgen stain
Ethyl green-Pyronin Y

Connective tissue (*section 4.2.2*)
Collagen fibers
Masson's trichrome
Mallory's trichrome
Gomori's trichrome

Elastic fibers
Verhoeff-Van Gieson
Weigert's Resorcin-Fuchsin
Orcein

Reticular fibers
Wilder (silver impregnation)
Gordon and Sweet (silver impregnation)

Minerals (*section 4.2.3*)
Calcium
Von Kossa
Alizarin red S

Copper
p-Dimethylaminobenzidine rhodamine (DMABR)

Pigments (*section 4.2.4*)
Iron
Prussian Blue (Perl's staining)

Melanin
Masson-Fontana silver
Schmorl's ferricyanide