9 Theory of histological staining

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Introduction

The physicochemical mechanisms of most histological stains are now understood. Detailed accounts and general overviews are to be found in the references and further reading at the end of this chapter. Histological staining methods from acid dyes to silver impregnation, involve broadly similar physicochemical principles. The present chapter aims to outline the major theories on common staining procedures and facilitate rational trouble-shooting if problems are encountered.

Key questions to consider when seeking to understand histological stains are:

- Why do *any* tissue components stain?
- Why do stained components *remain* stained?
- Why are *all* components not stained?

These questions can be answered for most stains, although some answers are complex. For instance, enzyme histochemistry, immunostaining and the PAS procedure involve biochemistry, immunochemistry and organic chemistry respectively. However, these methodologies are all influenced by selective uptake of stains and staining reagents into cells and tissues, and selective losses of stains from tissues. Which uptakes and losses occur depends both on binding equilibria and on rate factors.

Nomenclature note: *staining* always involves the visual labeling of some biological entity by attaching, or depositing in its vicinity, a marker of characteristic color or form. The *stain* is the marker, or the reagent used to generate the marker.

Why and how staining happens

Why are stains taken into the tissues?

Stain uptake is often due to dye-tissue or reagent-tissue affinities. In the physicochemical literature, to say a tissue component has a high affinity for a dye merely means there is a tendency for a stain to transfer from solution onto a section and this concept is used here. The magnitude of the affinity depends on every factor favoring or hindering this movement. The familiar stain-tissue attractions, including stain-solvent and stain-stain interactions, can be influential, as can solvent-solvent interactions.

This account initially assumes staining reaches equilibrium, and the consequences of this not being reached are discussed later. Moreover, uptake of dyes and reagents is often multistep in both space and time. Thus, a reagent may initially enter tissues due to coulombic attractions. Once inside it may form covalent bonds with some tissue grouping. The intensity of staining may also be influenced by stain solubility in solvent and tissue environments.

Various contributions to stain-tissue affinity are outlined in Table 9.1 and are discussed below. Practical staining processes commonly involve several such factors. However, as histologists and histochemists often emphasize reagent-tissue attractions as affinity sources, these interactions are discussed first.

Reagent-tissue interactions

Coulombic attractions have been termed salt links or electrostatic bonds, and have been the most widely

Table 9.1 Factors contributing to dye-tissue affinities

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

discussed reagent-tissue interactions. They arise from electrical attractions of unlike ions, e.g. the colored cations of basic dyes and tissue structures rich in polyanions such as phosphated DNA or sulfated mucosubstances (Lyon, 1991; Prentø 2009). However, binding of dye ions to an ionic tissue substrate also depends on charge magnitude, the amount of non-dye electrolyte in the dyebath, electrical repulsions between ions of similar charge, and swelling or shrinking of tissue substrates (Scott, 1973; Bennion & Horobin, 1974; Goldstein, & Horobin, 1974b; Horobin & Goldstein, 1974). These phenomena are relevant for all ionic reagents, not just dyestuffs. For example when using periodate as the oxidant in the PAS procedure, the periodate anions do not readily react with anionic polysaccharides, such as chondroitin sulfate (Scott & Harbinson, 1968). Moreover, even uncharged tissue substrates acquire an ionic character after binding ionic reagents, e.g. during staining of glycogen by the PAS procedure.

Reagent-tissue attractions not depending on isolated electric charges include dipole-dipole, dipole-induced dipole and dispersion forces; collectively described as *van der Waals' forces*. These occur between all reagents and tissue substrates. However, as extensively delocalized electronic systems favor larger dipoles and greater polarizability, van der Waals' forces are more significant when tissues or stains contain such moieties. Consequently, proteins rich in tyrosine and tryptophan residues, and nucleic acids containing heterocyclic bases, favor van der Waals' attractions. This is also true for the large aromatic systems of bisazo dyes and bistetrazolium salts, halogenated dyes such as rose Bengal and phloxine, and indoxyl and naphthyl enzyme substrates (Horobin & Bennion, 1973). For instance, van der Waals' attractions contribute substantially to stain-tissue affinity when staining elastic fibers which are rich in aromatic desmosine and isodesmosine residues with polyaromatic acid and basic dyes such as Congo red and orcein (Horobin & Flemming, 1980).

Hydrogen bonding is occasionally discussed in the biological staining context. This attractive interaction arises when a hydrogen atom lies between two electronegative atoms e.g. oxygen or nitrogen, though being covalently bonded only to one. Water is hydrogen bonded extensively to itself, forming the clusters important for the hydrophobic effect discussed below. This effect also applies to molecules carrying hydrogen bonding groups present in many dyes and tissue components. As water molecules vastly outnumber dye molecules, hydrogen bonding is not usually important for stain-tissue affinity when aqueous solvents are used. Exceptions arise when hydrogen bonding is favored by the substrate, as with collagen tissue fibers (Prentø, 2007). A related attractive phenomenon, halogen bonding (Metrangolo et al., 2005), may also contribute to staining affinity. This could explain strong staining seen with dyes such as eosin Y (4 arylbromo

substituents), phloxine (4 bromo plus 4 chloro), and other halogenated fluoresceins.

Covalent bonding between tissue and stain is yet another source of stain-tissue affinity. This process underpins the commonly used PAS reactive stains, as well as the historic Feulgen nuclear stains. The polar covalent bonds between metal ions in dyes such as hematein and tissue substrates are another possible example. Dye-tissue binding considered to involve such polar bonds is termed *mordanting*. However, this is of uncertain status since the characteristic staining properties of mordant dyes may have other, or additional causes. Unlike most cationic dyes used as biological stains, common cationic metal-complex dyes are strongly hydrophilic (Bettinger & Zimmermann, 1991) and resist extraction into alcoholic dehydration fluids (Marshall & Horobin, 1973).

Solvent-solvent interactions

A major contribution to stain-tissue affinity when using organic reagents or dyes in aqueous solution is the *hydrophobic effect*. This involves no stain-tissue attractions, but is the tendency of hydrophobic groupings (e.g. leucine and valine side chains of proteins; biphenyl and naphthyl groupings of enzyme substrates and dyes) in an aqueous milieu to come together, even though they were initially dispersed. This interaction occurs because water molecules are linked together by hydrogen bonds into transient clusters whose presence is favored by hydrophobic groups. Any process breaking clusters into individual water molecules occurs spontaneously, as this increases system entropy (cf. the second law of thermodynamics). Consequently, removing cluster-stabilizing hydrophobic groups from contact with water by placing them in contact with each other, is thermodynamically favored. Accounts of the hydrophobic effect are provided by biochemists, amongst others (Tanford, 2004). The effect becomes more important as substrate and reagent become more hydrophobic. Thus, when staining fats with Sudan dyes applied from substantially aqueous solutions, the hydrophobic effect provides major contributions to affinity. Although the phenomenon is sometimes termed hydrophobic bonding, no dye-tissue hydrogen bonds are involved.

Staining using Sudan dyes in non-aqueous solvents does not involve the hydrophobic effect. However, as described in chemical thermodynamics texts (e.g. Adamson, 2012), the tendency of a system to change spontaneously to maximize its disorder, and for *entropy* to increase, provides an explanation. Presence of dye in solvent and lipid constitutes a more disordered system than dye restricted to the solution. So dye disperses, and staining occurs. Such increases in entropy involving substrate and stain occur in all types of histological staining.

Stain-stain interactions

Dye-dye interactions can also contribute to affinity. Even in dilute solutions, dye molecules can attract each other, forming aggregates. In aqueous solutions this may be driven by the hydrophobic effect, but in both aqueous and non-aqueous solutions van der Waals' attractions between planar dye molecules occur. Dye aggregation increases with concentration, e.g. when high dye concentrations build up on tissue sections. With basic (cationic) dyes, such as toluidine blue, this occurs on substrates of high negative charge density e.g. sulfated polysaccharides in mast cell granules giving *metachromatic staining*. This color effect arises because dye aggregates have spectral properties different from the monomeric dye. That dye-dye interactions contribute to affinity in tissue sections was demonstrated quantitatively by Goldstein (1962).

Other cases where stain-stain interactions contribute to affinity include metallic nano and microcrystals generated by gold or silver impregnation (Uchihara, 2007), ionic metal sulfide precipitates formed in Gomori-type enzyme histochemistry, and the purple azure-eosin charge transfer complex produced during Romanowsky-Giemsa staining of cell nuclei (Horobin, 2011).

A minor anomaly

Some stains are not taken up by their tissue targets. In *negative staining* the shapes of structures are disclosed by filling or outlining them with a stain. Examples include demonstrating canaliculi in bone matrix using Schmorl's picro-thionine stain

and visualizing individual microorganisms using nigrosine.

Solubility, an unacknowledged factor

The solubility of stains and staining reagents is a key practical property. Thus, when staining fat with a Sudan dye, the upper limit of staining intensity is set by dye solubility in the lipid, and is further influenced by dye solubility in the staining bath solvent. Solubility is also critical for dye retention after staining, as discussed below. Solubility has complex causes but, in general, the stronger the reagent-reagent interactions, the lower the solubility. Physicochemical texts provide overviews of solubility e.g. Letcher, 2007.

Why is stain retained in tissue?

After removal from the staining bath, stain retention occurs if stains have a high affinity for tissue elements and/or low affinity for processing fluids and mounting media, or if stains dissolve in these latter solvents slowly. This is illustrated by the following examples of common stains.

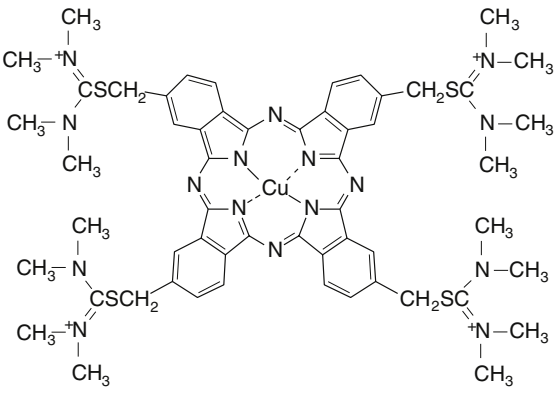
Ionic pigments, such as the Prussian blue generated in the Perls' method for iron, and the lead sulfide produced in Gomori-style enzyme histochemistry are virtually insoluble in solvents used in histotechnology. This is also true for microcrystals of metallic silver and gold produced by metal impregnation. Some organic pigments are less satisfactory. Azo dyes, formazans and substituted indigos produced as final reaction products in enzyme histochemistry have low solubilities in water, but may dissolve in hydrophobic media such as alcohols, xylene or polystyrene. In such cases, hydrophilic mounting media are required, and staining of lipid-rich tissue elements may be artifactual.

Solubilities of azodyes and formazans are sometimes reduced by in situ conversion to metal complexes. Other routine metal complex stains are the aluminum, chromium and iron complexes of hematein, and the chromium complex of gallocyanine. These complexes are poorly soluble in routine processing fluids and mounting media.

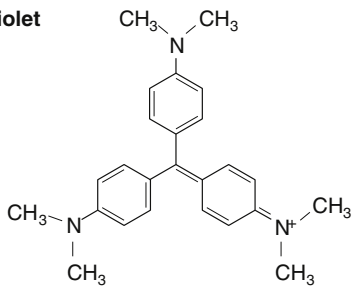
This contrasts with routine basic (cationic) dyes such as crystal violet or methylene blue, which freely and rapidly dissolve in the lower alcohols. Routine acid (anionic) dyes, such as eosin Y or orange G, are often less soluble in lower alcohols, as indeed are hydrophilic basic dyes with large aromatic systems, such as alcian blue. Non-ionic dyes e.g. Sudan stains, are soluble in common dehydrating agents and clearing solvents, as well as in resin mountants. Structures of exemplar hydrophilic and lipophilic basic dyes are shown in Fig. 9.1.

Consequently, sections stained with routine basic dyes must be dehydrated by either passing rapidly through the alcohols, using non-alcoholic solvents or by air-drying. Dehydration is less critical with acid dyes. Sections stained with acid or basic dyes are

Alcian blue 8G



Crystal violet



Dye	Ionic weight	Log P
Alcian blue 8G	1380	–9.7
Crystal violet	372	+1.9

Fig. 9.1 Structural formulae of two widely used basic dyes, plus numerical descriptions of certain of their physicochemical properties. Log P is the logarithm of the octanol-water partition coefficient.

usually mounted in non-aqueous media which do not extract dye. Alternatively, dyes may be immobilized, e.g. by formation of phosphotungstates in the modified Schmorl's method or iodine complexes in the Gram stain. Non-ionic dyes must be mounted in aqueous media.

Why are stains not taken up into every part of the tissue?

The phenomenon of selectivity is crucial for special stains and histochemistry. Even routine oversight stains e.g. hematoxylin and eosin (H&E), Papanicolaou and Romanowsky-Giemsa distinguish nuclei from cytoplasm. The various factors controlling selectivity are discussed below.

Numbers and affinities of binding sites

Both these factors separately influence staining but are not readily distinguished, except by quantitative investigation. Consequently, they are discussed here without distinction.

To understand practical stains it must be appreciated that non-staining of the "background" tissue is as important as staining of the "target" in the material. Sudan dyes provide an example as they have high affinity for fat, but low affinity for the surrounding hydrated proteins. Alternatively, one may consider staining systems in which covalent bonds are formed. Reagents usually give colored products only with a limited range of tissue chemical groupings. Thus, the acid hydrolysis–Schiff reagent sequence of the Feulgen nuclear technique gives magenta derivatives only with DNA. Other examples are provided by traditional anionic dye-cationic dye pairs e.g. H&E, Papanicolaou and Romanowsky stains. The negatively charged acid dyes have high affinities for tissue structures carrying cationic charges i.e. proteins under acidic conditions. However, they have low affinities for structures carrying negative charges e.g. those rich in sulfated glycosaminoglycans or in phosphated nucleic acids. The opposite is the case for basic (cationic) dyes. This produces two-tone staining patterns in which cytoplasm contrasts with nuclear material.

Practical staining protocols maximize selective affinities. Acid (anionic) dyes are applied from acidic solutions, when proteins carry an overall positive charge. Basic (cationic) dyes are applied from neutral or acidic solutions, since under alkaline conditions proteins carry an overall negative charge and also bind basic dyes. Affinities are also influenced by varying inorganic salt concentrations. The selectivity of aluminum-hematoxylins, the critical electrolyte concentration methodology (Scott, 1973) and Highman's and Puchtler's Congo red stains for amyloid all depend on control of electrolyte content.

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 (Goldstein, 1962, Goldstein & Horobin, 1974a). 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, so increasing the ease of practical control.

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. Enzyme histochemistry provides further examples. When incubating at low pH, hydrolysis of an organic phosphate is rapid in tissues containing acid phosphatases but slow in structures containing alkaline phosphatases.

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 (Kiernan, 2002; Uchihara, 2007).

Artifacts abound in rate-controlled methods. Any factor influencing rate of reagent entry or loss e.g. section thickness, presence of cavities in the tissue, temperature and stirring of reagent solutions can alter staining results.

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 (reviewed by Pearse, 1968).

How is staining influenced by tissue fixation?

Fixation is carried out to reduce the non-vital tissue autolysis causing morphological changes, and to prevent losses of some tissue constituents in the processing and staining solutions. This chapter only discusses the influences of fixation on staining.

A given substance may be retained in the specimen to different extents by different fixative agents, and nothing can be stained if it is not retained. For example, many lipids are well preserved after fixation in osmium tetroxide or dichromates, but are poorly preserved after formalin fixation. However, lipids are actively extracted during alcoholic or acetone fixation. Thus, staining lipids after alcoholic fixation is ineffective.

Retention of substances to be stained is necessary, but mere retention may be insufficient. For example, although glutaraldehyde retains more protein than other fixative agents, its use in immunostaining and enzyme histochemistry is limited, even though most antigens and all enzymes are proteins. Chemical reactions insolubilizing proteins also modify haptenic and enzymic activity. Alcohol and acetone, although poor at retaining proteins, are also poor at destroying the activity of whichever antigen or enzyme is retained. Both retention and reactivity of substances affect staining, and both may be fixative dependent.

Fixation also has more subtle influences on staining patterns, acid and basic dyeing provide instructive examples. As shown by Singer (1952), such staining is enhanced by fixative-induced protein denaturation, but the acidophilia-basophilia balance of a tissue is often altered e.g. formalin and osmium tetroxide usually reduce tissue acidophilia, whilst acidic dichromate solutions usually increase tissue acidophilia (Baker, 1958).

What are the effects of specimen geometry on staining?

Here the 'specimen' refers to the biological material in contact with the staining solution, e.g. a dewaxed section or a cervical smear. Such specimens not only have breadth and width, but also thickness and three-dimensional morphology. These features, even at a scale of a few μm or less, influence staining patterns.

Simple geometrical influences

Typically, thin specimens achieve staining equilibrium faster than thick. Specimens with irregular surfaces are stained faster than smooth surfaces, and dispersed cells faster than uniform slabs. Consequently,

in any staining procedure, dispersed specimens such as smears or dabs require shorter staining times than sections of similar cells cut from a solid tissue. Moreover, cryosections usually have irregular surfaces and stain faster than smoother paraffin sections. Resin sections have even smoother profiles than paraffin sections and stain slower, especially when resin remains in situ.

In systems with rate-influenced staining mechanisms, such features modulate selectivity. Some trichrome stains require shorter staining times with cryo-sections than with paraffin sections to avoid overstaining by the higher ionic weight dye.

Effects of more complex specimen geometry

Complex geometries can arise when preparing smears from epithelia. These often contain multicellular clumps of cells, as well as monocellular dispersions. Cells in the middle of such clumps are less accessible to stains than the peripheral cells. Consequently, in rate-influenced methods, such as the Papanicolaou and Romanowsky-Giemsa stains, centrally situated cells can be overstained by the smallest dye present (see Boon and Drijver, 1986, Plates 6.4 and 24.4).

A section's profile may be influenced by fixation. Coagulant fixatives such as Carnoy's fluid tend to shatter cells and tissues, generating more dispersed specimens, whilst fixatives such as formalin give more integral forms (see Horobin, 1982, Fig. 14). Consequently, a rate-influenced trichrome stain which gives the correct color balance when applied to formalin-fixed tissue may overstain with large, collagen binding dyes if applied without modification to material fixed in Carnoy's fluid.

A modification of section geometry induced by microtomy is "chatter". This artifact, related to poor section cutting, produces sections comprised of alternating thick and thin strips. Staining may then generate alternate strips of strong and weak color intensity or, with some trichromes, alternate strips of varying color.

The size of biological structures relative to section thickness can also be significant. Consider secretory granules with diameters much larger or much smaller than the section thickness. All large granules will be sliced through with their contents exposed on a surface of the section, whereas some small granules will be intact and enclosed within the section. This 'access' influences accessibility of larger stains, e.g. macromolecular-labeled antibodies where the 'two types of secretion granule' sometimes reported in immunostaining studies can represent intact versus sliced granules. Such effects are even more pronounced in resin sections.

Geometrical complexity also arises from swelling of cell and tissue components in staining solvents. Materials rich in glycosaminoglycans, e.g. mucus and cartilage matrix, swell markedly in aqueous solutions but collagen fibers swell grossly at extremes of pH. Swelling can increase rates of staining of these structures compared to other material. This contributes to the selectivity of aqueous alcian blue for mucins, with nuclear staining typically being absent after short staining times, and to the selectivity of strongly acidic picro-trichrome stains for collagen fibers. Alcohol does not induce swelling, this may partially account for changes in staining pattern seen when a dye is used from alcoholic rather than aqueous solution. Luxol fast blue, for instance, stains myelin selectively from aqueous solution, but from alcoholic solution gives selective staining of collagen fibers. Such effects are often more marked in resin sections.

What are the effects of resin embedding on staining?

Resin embedding involves infiltration of biological material with a reactive monomer, most commonly an acrylate or epoxide. Subsequent polymerization yields a block of resin enclosing the specimen. Methyl methacrylate resin (MMA) is usually removed prior to staining, after which staining patterns resemble those of paraffin sections. However, glycol methacrylate (GMA) or epoxy resins typically remain in the sections during staining, resulting in different staining patterns to those seen in paraffin wax or cryosections. This is also the case for specimens embedded in a preformed polymer, nitro-cellulose (celloidin). Resulting staining changes have various causes.

Resins can act as stain excluders by obstructing penetration of staining reagents. Resin cross-linking reduces penetration even further. However, resin embedding involves more than mere reduced staining. Resin infiltrates biological specimens unevenly, with dense and hydrophilic structures being poorly infiltrated. For instance, in GMA-embedded specimens, dense or hydrophilic secretion granules are poorly infiltrated, and so stain readily. If the surrounding cytoplasm is well infiltrated with resin, granules may stand out more clearly than in paraffin sections.

Resins can act as stain binders e.g. GMA sections may give background staining with lipophilic dyes such as aldehyde fuchsin. This occurs because the resin itself is slightly lipophilic (Horobin et al., 1992). GMA can also strongly bind to dyes of moderate size, e.g. alum hematoxylins or eosin. These enter the resin and decrease the permeability of the polymer network, so trapping the dye. However, such dye can sometimes be removed by differentiating in plasticizing solvents such as ethanol.

Stain chemistry influences staining patterns: small reagents diffuse rapidly through resins and methods developed for paraffin or cryostat sections can usually be used without modification. When working with routine GMA embedding media, 'small' means <550 Daltons (Da), and includes such common substances as methylene blue, naphthyl phosphate and Schiff reagent. However, large reagents may be totally excluded from resin, restricting staining to resin-free structures. With routine GMA resins, 'large' reagents are those with sizes >1000 Da, e.g. alcian blue, Sirius red and labeled antibodies. The phenomenon of stains binding to lipophilic embedding media noted above occurs only with lipophilic reagents (Horobin et al., 1992).

Some dyestuff properties

General influences of dye chemistry on staining

When physicochemical features of dyes which influence dye-tissue affinity and staining rates are described numerically, systematic correlations can be demonstrated between dye chemistry and staining outcomes. Significant physicochemical parameters include electric charge, the overall size (as represented by ionic or molecular weight) and the hydrophilic/lipophilic character (modeled by the log P value, i.e. the logarithm of the octanol-water partition coefficient). To appreciate the advantages of numerical parameters, see Fig. 9.1, where chemical information concerning two widely used basic dyes is presented in two modes, graphical and numerical.

When considering the relative sizes of dyes, information provided graphically by structural formulae is satisfactory. One can see that alcian blue is a much larger dye than crystal violet. The fact that the staining pattern of alcian blue is highly dependent on staining time (Goldstein & Horobin, 1974a) is therefore not surprising. Unfortunately, the relative hydrophilic/lipophilic character of the two dyes cannot be easily assessed by visual inspection of formulae. However, the log P values of the dyes are clearly different; negative values imply hydrophilicity and positive values lipophilicity. In keeping with this, during alcohol dehydration, sections stained with alcian blue lose no dye, whereas crystal violet is easily lost.

Prediction of stain behavior usually requires consideration of several dye properties. Detailed discussion here is inappropriate, but note that quantitative structure-staining correlations based on such structure parameters can illuminate diverse issues in histotechnology from the staining mechanisms of trichromes (Horobin & Flemming, 1988), to assessment of effects of resin embedding on histochemical staining procedures (Horobin et al., 1992). For overviews see Horobin (2004, 2010).

Effects of dye impurities on staining

Most dye lots used as biological stains are impure. Some contain colored substances not named on the label, or may even lack the named dye. If the colored material is as stated, it may be diluted by colorless materials, e.g. dextrin or an inorganic salt. Moreover, dyes which are pure when purchased may degrade on storage, after being made up into a staining solution, or during staining.

Impurities influence staining in two ways. Firstly, they may alter staining intensity, usually reducing

staining, although occasionally more intense color is seen. Secondly, impurities may change staining patterns by altering the nature and mechanisms of such effects depending on the type of impurity, the staining procedure and the tissue substrate. Unfortunately there is no simple way to identify, and so avoid, such impure products. A practical tip is to purchase dye lots certified by the *Biological Stain Commission*. These have been tested in the Commission's laboratory, and meet purity and staining efficacy criteria. Surprisingly, Commission certified dyes are on average no more expensive than non-certified dyes. For an example of the benefits of certified dyes, see Henwood (2003). Another practical tip is to retain samples of effective dyes. Then, faced with an unexpected staining pattern, to stain the sample with the known effective dye sample, thus ensuring validation. In short, if the known standard dye works, then there may be problems due to dye impurity in the new batch.

If you have an impure dye sample, the most useful advice is to buy another batch of dye, preferably Biological Stain Commission certified. If analysis or purification does prove necessary, an extensive analytical literature may be accessed for individual dyes via the monographs of the 10th edition of *Conn's Biological Stains* (Horobin & Kiernan, 2002), or more generally via an earlier review article by the present author (Horobin, 1969).

Dye nomenclature

Names of individual dyes, and terms used to describe the dye properties, are sometimes inconsistent and/or often confusing. Dyes are complex molecules, nearly all having trivial names which do not explicitly describe their structures. Most dyes used as biological stains were first manufactured as textile dyes when each manufacturer gave a dye their own trade name. A biologist may therefore say 'Use Congo blue', to which his colleagues reply 'But we haven't got any of *that*'- they have, but on their shelves it is labeled trypan blue. Even worse is the surfeit of suffixes. Sometimes these are merely flourishes of a copywriter's pen, so pyronines G and Y are synonyms. Sometimes suffixes indicate dye content, and a standard product may be labeled 'A 100' whilst a grade containing a higher content of dye is termed 'A 150' or merely 'A extra'. Sometimes however, suffixes indicate substantial chemical differences e.g. rhodamines B and 6G describe zwitterionic and cationic dyes respectively.

To reduce confusion, industrial dye users established the *Colour Index* (Society of Dyers and Colourists, 1999). Dyes are given unique code numbers, the *Colour Index* or C.I. number and code names. Thus eosins G, WG and Y are identified as a single dye, C.I. 45380, Acid Red 87; whilst eosin B is a chemically different dye, C.I. 45400, Acid Red 91.

Dyes synthesized for biological staining are named equally idiosyncratically. A traditional example is Gomori's aldehyde fuchsin, and a recent one YOYO-1. Since these products are not of industrial significance, most do not have a *Colour Index* entry. If concerned/confused in such cases one may peruse *Conn's Biological Stains*; see Lillie (1977) and Horobin and Kiernan (2002) for the 9th and 10th editions, respectively. The earlier edition emphasizes traditional stains, the later edition fluorescent probes.

Various terms used to classify dyestuffs are given in Table 9.2. A few comments follow on topics which are sometimes confused in the histochemical literature. *Acid* and *basic* dyes are not acids and bases but salts whose colored species are anionic and cationic respectively. *Neutral* dyes are not non-ionic, but salts in which both the anion and cation are dyes. *Vital stains*, used to stain living cells, are nowadays often called *fluorescent probes* or *biosensors*. One should note that all dyes can be given multiple descriptors. Thus, alcian blue 8G is a *synthetic, basic* dye, structurally a *metal complex*, though not a *mordant dye*, of copper with *phthalocyanine,* substituted by *thioguanidinium groups*, and is routinely used as a *mucin stain*.

Problem avoidance and troubleshooting

Avoiding problems and recognizing errors and achieving a correction are perennial laboratory concerns. The typical strategies are discussed below.

Table 9.2 Some descriptive terms used in classifying dyestuffs used as biological stains

Categories of terms	Examples of terms (and of dyes)
Describing the origins of a dye	Natural (hematoxylin and carmine), synthetic or aniline (almost any other)
Describing physicochemical properties of a dye	Fluorescent (acridine orange), leuco (leuco-methylene blue), metachromatic (toluidine blue), neutral (azure-eosinate)
Giving some kind of description of the dye's structure	Azo (orange G), metal complex (aluminum or iron complexes of hematein), xanthene (pyronine Y)
Describing the dye's usage in biological staining	Fat (oil red O), fluorescent probe (YOYO-1), mucin (alcian blue)
Describing the dye's usage in textile dyeing	Acid (eosin), basic (safranine), direct (Congo red)
Describing the supposed mode of action of the dye	Mordant (gallocyanine chrome alum), reactive (mercury orange)

Strategies for avoiding problems, minimizing the need for troubleshooting

Issues concerning staining procedures

- Use stains compatible with the fixative and embedding medium. *Case example*: water-miscible resin sections do not allow selective staining of elastic fibers with aldehyde fuchsin.
- Use a routine, preferably standardized, staining protocol. *Tip*: see the listing of such protocols at the back of Horobin and Bancroft (1998).
- Use controls proactively to identify problems, not just retrospectively to investigate mistakes. *Tip*: retain samples of stain lots to use if you suspect stain impurity.
- Consider whether you have the necessary skills and knowledge, or if not, who could mentor you. *Tip:* silver stains are tricky; read Kiernan (2002) first, and expect problems with their use.

Issues concerning staining reagents

- Obtain reliable stains and reagents. *Tip*: use Biological Stain Commission certified dyes, which are usually less impure but no more expensive.
- Ensure stains remain reliable. *Tips*: store Schiff reagent in a gas-tight container and dye solutions in lightproof containers.

Cues for recognizing errors - before mistakes can be rectified they must be noticed

- Stain or staining solution is not as expected in terms of color, solubility or stability. *Case example*: some alcian blue samples dissolve, but then precipitate from solution within an hour or less.
- The expected structures stain, but only weakly. *Case example*: unexpectedly weak staining of calcium by alizarin red S can result from aqueous fixatives extracting tissue calcium ions.
- Color of staining is unexpected. *Case example*: excessively red staining seen with Gomori's trichrome may arise from insufficiently acidic staining solutions.
- Unexpected structures stain. *Case example*: granular material stained by the Feulgen nucleal procedure may be carbonate deposits.
- Nature of the staining is unusual. *Case example*: if differential staining of Gram positive and negative organisms is poor, the preparation may be too thick.
- Remember there are always *other* problems! *Case examples*: loss of sections from slides in the Grocott hexamine silver method for fungi due to overheating, and black deposits on slides and sections in the von Kossa procedure due to contaminated glassware.

Once an error has been noticed and a plausible cause identified, corrective action can be taken. A variety of practical problem-solving suggestions for a range of routine and special histopathology stains is provided in Horobin and Bancroft (1998).

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Further reading

Accounts of histotechnology and histological staining rarely describe the physicochemical unity underlying the technical diversity of the various staining technologies. Published protocols are legion, critical reviews and summations rare. Encyclopedic texts such as this one, and earlier works such as those of Lillie (1965), Pearse (1968), and Sheehan and Hrapchak (1987) summarize a remarkable amount of information, and provide extensive bibliographies. Some staining manuals integrate theoretical background with procedural information, e.g. Chayen and Bitensky (1991) and Kiernan (2015). A few authors have provided systematized accounts of staining methods from a modern physicochemical perspective; for instance, Horobin (1982, 1988), Horobin and Bancroft (1998), Lyon (1991), and Prentø (2009). Some classic works can also be recommended. Read Baker (1958) for his early integrative account and his elegant English. Read Lillie (1965) for his hard-won personal experience and historical long view. Then read Mann (1902) to be astonished at why it took us so long to follow up his experimental investigations and mechanistic insights. Several modern texts concerning dyestuffs are available including that of Zollinger (2003) which does, unusually, include a substantial section explicitly discussing biological stains and staining.