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Review

Histological stains and their application in teaching and research

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ABSTRACT

This review aimed to highlight the historical perspectives focused on histological stains and their application in teaching and research. While early histologists used indigenous substances like alizarin, carmine, and saffron to stain tissues, modern histologists utilize techniques that study cellular organelles and substances within tissues. Despite the advent of new staining techniques, the modification of old ones, and decrease in the use of natural dyes in the 20th century, some of the stains used in the past are still very much in use. These techniques are used in teaching and research for the demonstration and display of the different substances within tissues. These include the Giemsa stain for parasites and fungi, the Trichrome stain for collagen, oil red O stain for lipids, periodic acid Schiff for glycogen, Crystal violet for Nissl granules, Golgi stain for neuronal cells, as well as routine hematoxylin, and eosin (H&E) for a general histology. Histological stains can be used to teach histology, cellular pathology, microbiology, and parasitology. They can also be used to study the rate of progression of disease or the healing process of diseased conditions.

Key words: Eosin, haematoxylin, oil red o, research, stains, teaching

INTRODUCTION

Histology entails the study of the microscopic structure of tissues with the aim of assessing their function [1]. Light microscopes use transparent glass lenses and visible light while electron microscopes use a beam of electrons and an electron detector to magnify the images taken of the tiny cellular structures [2,3]. Microscopes can distinguish between structures based on the order of the wavelength of visible light (390 and 700 nanometers) or that of the electron beam (100,000 times smaller compared to visible light). Light microscopes cannot be used to observe things smaller than a few hundreds of nanometers but electron microscopes have a higher resolution due to the short wavelength and can reveal and distinguish between the structures of smaller objects [4]. Despite the inability of light microscopes to magnify structures <100 nanometers, they preserve the full color of images and can be used to study wet specimens without requiring a vacuum. Hence, light microscopes still remain a widely used instrument in histology [5].

Contrast media are used to distinguish between different structures. They are in the form of stain absorbed by some cells or cellular organelles to enhance imaging and they can be used to demonstrate the presence or absence of certain products of metabolism within cells and tissues [6]. The stages in routine histological techniques for the preparation of tissue before staining are as follows: fixation, dehydration, clearing, embedding, sectioning, and rehydration [7]. A routine hematoxylin-eosin (H&E) stain is commonly used in histology while special stains are used in special cases to demonstrate the presence of collagen fibers, copper, iron, and fat droplets within tissues. Special stains include Periodic Acid Schiff (PAS) for carbohydrate, Masson's trichrome for collagen, the Sudan stain and oil red O for adipose tissue, Perl's stain for iron, and Rhodanine stain for copper [8–10]. Oil Red O is mostly implemented on frozen sections, thus it is not commonly useful in routine preparation [11]. The process of preparing tissues for Oil Red O staining only involves freezing and sectioning. Special stains depend on some basic chemical reactions to visualize, identify, and distinguish between tissues, cells, organelles, carbohydrates, fats, minerals, enzymes, and microorganisms [12]. These special stains improve tissue contrast, demonstrate vital cellular organelles, and help in disease evaluation/diagnosis and the identification of pathogens. The current review was aimed at highlighting the historical perspectives of histological stains and their application in teaching and research.

HISTORY OF HISTOLOGICAL STAINING

Early histologists used indigenous substances like Tyrian purple, alizarin, carmine, saffron, and indigo to stain tissues. Some of these dyes were adapted from the textile industry [13]. Later techniques were devised

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to study cellular organelles through the use of staining techniques such as silver nitrate, cochineal, and carmine. Cochineal, one of the early dyes, was used by 17^{th} century scientists such as Leeuwenhoek and Hooke to stain tissues. Sir John Hill, an English microscopist, used cochineal in an alcoholic solution to stain wood fibers in 1770[7]. In 1825, Raspail demonstrated the presence of starch in plant cells using iodine. A famous French physiologist, Claude Bernard, used iodine to stain a liver tissue substance that was later identified and called glycogen in 1849[14]. In later years, some German scientists used carmine staining to describe tissue structure. They included Ehrenberg in 1838 and Corti in 1851[15]. Joseph Von-Gerlach was assumed to have initiated the microscopic staining protocol in 1858 when he stained and demonstrated cerebellum with ammoniacal carmine[7,16].

The creation of the aniline dye industry in 1856 led to the production of new dyes useful to histopathology. These include the synthesis of aniline purple by Henry Perkin in the same year and the synthesis of basic fuchsin, aniline blue, eosin, and methylene blue in 1858, 1862, 1871 and 1876 respectively[17]. Perl's Prussian blue method for hemosiderin (iron) was published in 1867. The technique involves the unbounding of the covered ferric iron in tissue and combining it with potassium ferocyanide to form a Prussian blue color, ferric ferocyanide[18].

Hematoxylin is a natural dye that has been in use ever since its discovery from the logwood tree. The protocols for the preparation and use of aqueous hematoxylin stains was published by Cook in 1879 using an alum/copper sulphate extraction procedure to remove the hematoxylin from the logwood[19]. Wissowzky devised the hematoxylin and eosin staining procedure in 1876 which entailed nuclei staining with alum hematoxylin, differentiation with dilute acid alcohol followed by bluing in running water, and staining the cytoplasm with eosin[20]. In 1882, Robert Kock devised a method for the demonstration of *Mycobacterium tuberculosis* in a blood smear sample. The method involves the treatment of the smear with a mixture of potassium hydroxide and methylene blue followed by the differentiation of methylene blue with concentrated Bismarck brown giving the bacterium a blue color with brown cells and nuclei. In 1892, Dimitri Romanowsky described a technique for staining parasites in blood smear. His method employs the use of methylene blue and its products of oxidation in combination with eosin to stain the blood smear, producing a red nuclei[13,21]. The method was modified by Wright in 1902 and Giemsa in 1902 and these methods have significantly improved the staining of parasites.

Before the introduction of Sudan III in 1896, tissue lipids were demonstrated with osmium tetroxide[22]. The lipid reduces osmium tetroxide and producing a black coloration. Subsequently, other lipid stains such as Sudan IV, oil red O, and Sudan black were introduced in 1901, 1926, and 1935 respectively[22]. The discovery of the trichrome technique for muscle and connective tissues in 1900 was another significant event in the history of histological stains[23]. Other methods for the demonstration of connective tissues were described by Frederick Verhoeff in 1908 (elastin stain) for elastic fibers, Pierre Masson in 1926 (Masson's trichrome method) for elastic fibers, and George Gomori in 1950 (aldehyde fusion technique and the one-step trichrome method) for mast cells and elastic fibers. Additionally, Harold Steedman in 1950 (alcian blue) proposed a method for acid mucin and Kluver and Berrera in 1953 (luxol fast blue) for myelin[24,25]. The demonstration of amyloid in tissue sections was earlier described by Benhold in 1922 with Congo red stain. Five years later, Divry and Florkin also contributed to the technique of identifying amyloid in tissue sections by demonstrating the green birefringent effect of Congo red stain on amyloid[26,27]. In the early 1980s, Richard Allen medical industries try to standardize the technique of microscopic slide preparation by providing a special management system. They also produced fixatives, processing chemicals, wax, and stains[26]. The protocols for H & E staining were also adjusted for better contrast in pathologic tissues. The program was not widely accepted due to the variations when preparing the H & E stains[27]. Despite the advent of new staining techniques and the modification of the old ones in the 20^{th} century, some stains used in the past are still very much in use for the demonstration of amyloids, carbohydrates, and lipids in tissues[26].

APPLICATIONS OF HISTOLOGICAL STAINS

Staining is a sequence of procedures undertaken when preparing tissues to highlight important features within the tissue with either natural dyes that adhere to some cells and tissues or chemical reactions that result in the formation of precipitates. Staining also enhances tissue contrasts[6]. Staining is generally used in medicine for the analysis of tumors and pathologic changes within tissues due to disease or injury[28]. Improvements in the staining methods through molecular biology techniques, histochemistry, and immunological techniques have simplified the study of tissues[29]. Histological stains are the dyes

or substances used to enhance the contrast in tissues before microscopic examination. They include the routine H & E stain, Crystal violet stain, Gomori, and Masson's trichrome among others[28].

Hematoxylin and Eosin stains

Hematoxylin is a naturally occurring basic dye that is generally used in histology to stain the nuclei blue. Eosin is an acidic dye that principally binds to proteins and stains the cytoplasm, giving it a pinkish colour[13,30]. Hematoxylin is unable to stain unless it is oxidized to hematein (ripening). The ripening can be either slow and spontaneous by exposing hematoxylin to atmospheric oxygen or rapid by adding oxidants for example, sodium iodate (Gill) or mercuric oxide (Harris). Gill hematoxylin formulations use Aluminum sulfate as a mordant, a technique recommended by Baker[30]. The mordant gives Gill's hematoxylin the ability to stain mucin. Eosin Y (eosin G or Bromo acid) is the source of eosin stains (*eos* means dawn while Y means yellowish)[31]. H & E is the most common staining technique in histology. It shows the general organization of tissues, permitting the differentiation of structures to identify normal, degenerated and/or inflamed cells[19]. H & E can be used to stain paraffin and frozen sections as well as clinical and cytological specimens like urine, vaginal smears, and sputum. H & E stains can demonstrate the general structure of a tissue, making it the most common stain used when teaching microscopic anatomy and for research purposes. When tissues are stained with H & E, the nuclei and chromatin appear dark blue, cytoplasm appears red-orange with eosin Y and red with both eosin B and erythrosine B, while collagen, elastin, and erythrocytes appear yellow-orange with eosin Y and red-orange with both eosin B and erythrosine B[1].

Giemsa stain

The Giemsa stain permits the differentiation of cells present in hematopoietic tissues as well as allowing the detection of parasites and fungi. It was discovered by Gustav Giemsa, a German bacteriologist in 1904[32]. The Giemsa stain is the gold standard for detecting malaria parasites in blood smears[33]. The stain gained popularity in clinical microbiology because it is capable of revealing the morphological variances of *Plasmodium* species in infected erythrocytes[32]. The Giemsa stain can be used in cytomegalovirus infection to highlight inclusion bodies (owl's eye appearance) and to demonstrate giant proerythroblasts for the diagnosis of Parvovirus B19 infection. It can also stain microorganisms like Filaria, *Pneumocystis*, *Trypanosoma, Histoplasma*, and *Leishmania*[34,35]. Because this stain is used in the detection of microorganisms, it has a widespread application including the diagnosis of different infectious agents as well as teaching and research in microbiology, parasitology, virology, and public health[34]. In blood smears, red blood cells (RBCs) infected by *Plasmodium* species stain red[33]. In tissue sections, nuclei appear blue, the cytoplasm appears pale blue, and erythrocytes appear yellowish pink[36].

Trichrome stain

The trichrome stain consist of three dyes that stains the nuclei, cytoplasm, and collagen differently[37]. The three common trichrome stains are Masson's trichrome, the Van Gieson stain, and Gomori's one-step trichrome (GOT). Masson's trichrome stain consists of serial staining with iron hematoxylin, Biebrich scarlet, and aniline blue and/or light green to stain the nuclei black, cytoplasm and muscles red, and collagen blue or green respectively[37]. In un-decalcified bone sections, the bone matrix and osteoid border appears red, connective tissue appears pink, while synthetic bone substitutes appear bright green. In GOT, all dyes are present in a single solution together with glacial acetic and phosphotungstic acids. The red color here is imparted by chromotope 2R[31]. In this method, the appearance of the tissue components in paraffin embedded and frozen sections differ. For paraffin embedded tissues, collagen and mucin appear green, muscle fibers, cytoplasm, and keratin appear red, while nuclei appear blue/black[31]. In frozen sections, muscle fibers appear green, interstitial connective tissue appear light green, mitochondria appear red, myelinated nerve twigs appear red, and nuclei appear blue. The mechanism of the Van Geison stain is not clearly understood. However, it stains tissue components differently[38]. Here, collagen appears red while the nucleus appears black. Trichrome stains are utilized to evaluate fibrosis in tissues. It provides vital information on the stage of disease by providing a clear contrast between collagens and the surrounding connective tissue and cells[38]. It can be used to study the effect of drug and therapeutic agents on collagen fibers and to stain pancreatic beta cells to differentiate between the types of diabetes in experimental animals. Thus, it has applications in research and learning in the areas of cellular pathology, endocrinology, and experimental toxicology[39,40].

Oil red O stain

Oil red O and Sudan stains belongs to the polyazo group of dyes[41]. Oil red O stain can demonstrate neutral fat in different tissues to diagnose certain disease conditions associated with the deposition of fats within tissues[41]. The Oil Red O stain principle is based on its solubility in neutral fats compared to other solvents (alcohol)[42]. Oil Red O stains are best implemented on frozen sections because the tissue fats are removed by the xylene, alcohols, and/or paraffin. Therefore, Oil Red O stained tissues are used to demonstrate fatty tissues when teaching microscopic anatomy and pathology to medical, dental, and paramedic students. Oil Red O can be employed in cytology to differentiate liposarcoma from other mesenchymal tumors[42]. Oil Red O stained slides can be visualized under light and fluorescent microscopes where fat appears red and nuclei blue[43].

Crystal violet stain

Crystal violet (CV) is a cationic textile dye and biological stain. It is carcinogenic, hence the need to remove it from the wastewater o prevent environmental exposure[44]. Various methods such as adsorption, coagulation, precipitation, and membrane filtration have been used for dye removal from aqueous systems. Due to the low biodegradable nature of CV and its high stability in the effluent, dye removal through biological treatment was proven to be less efficient[45]. CV is used to stain Nissl substances. Because Nissl bodies may dissolve and disappear in certain pathological conditions, their presence or absence in the brain tissue is useful in the diagnosis of neurodegenerative disorders[45]. Crystal Violet is an acidotropic compound that accumulates in acidic organelles such as the lysosome where Nissl substances appear dark blue to purple[46].

Periodic Acid Schiff (PAS)

The periodic acid Schiff method was described independently by McManus[47], Hotchkiss[48], and Lillie[49]. The chemical basis of the PAS reaction is due to the ability of periodic acid to oxidize glycols into two molecules of aldehyde[50]. A pararosaniline adduct that is released after the reaction with Schiff's reagent stains all glycol-containing cellular components[48]. The PAS stain is used for the demonstration of mucopolysaccharides and fungi in tissue sections[51]. Together with diastase (α-amylase) digestion, it is used to diagnose glycogen storage disease[18]. PAS positive substances (glycolipids, glycogen, collagen fibers, neutral muco-substances, phospholipids, and basement membranes) appear pink, red, or purple while the nuclei appear blue. If α-amylase or diastase is used as a negative control, glycogen is removed, leaving the plasma membrane to appear pink[52].

Golgi stain

The staining of neuronal cells using silver impregnation where silver nitrate and potassium dichromate are used to stain the neurons was discovered by Camillo Golgi in 1873 and modified by Santiago Ramón Cajal in 1888[41]. The method is useful for studying the general morphology of glial cells and neurons in the central nervous system[53,54]. Golgi's method became very important when the structures that were previously described by Golgi as artifacts turned out to be dendritic spines in the studies of Cajal who was the first to describe the structure as small thorns that protrude from the dendrites of Purkinje neurons. In this way, he identified the real value of the Golgi method[55]. Since the discovery and modification of the Golgi method, several morphological studies have used the application of this method to categorize different kinds of neurons and to describe the functional areas of the brain[56]. There have been several modifications of the Golgi method. The Golgi–Cox technique is used to demonstrate dendritic arborization with little interference from the background structures. It was developed by Cox in 1891 and is comprised of specimen impregnation, tissue protection, and color development[57]. Other modifications to the Golgi method include the rapid Golgi method described by Cajal and De Castro in 1933. In this method, osmium tetroxide is used as a post-fixative to complement the Golgi stain[58]. The Golgi–Kopsch method is where glutaraldehyde and formaldehyde is used in place of osmium tetroxide as described by Kopsch and Colonnier[59]. In the Golgi method, 4–5 mm of nervous tissue are dipped in a solution containing 1 g of osmium tetroxide and 2.4% potassium dichromate for 1-7 days. The tissues are rinsed in a 0.75% aqueous silver nitrate solution until the brown precipitate disappears. After rinsing, the tissues are allowed to stay in the 0.75% silver nitrate solution for 1–2 days at room temperature. Subsequently, the tissues are embedded in paraffin, sectioned, cleared in xylene, and mounted in DPX[60]. Through the Golgi method, the nerve cells and processes appear as a fine, opaque precipitate with the neurons and neuroglia cells visible against a transparent or yellowish background[61].

CONCLUSIONS

Histological stains are used in microscopic anatomy to study the general architecture of tissues and to

demonstrate different substances such as carbohydrates, mucin, fats, collagen, and reticular fibers in tissue sections. They are also used to identify whether there are microorganisms present in blood smears, body fluids, fecal matter, and tissue sections. Because of the specificity of some histological stains, they can be used to diagnose disease conditions and to monitor the progress of a disease. Additionally, they are used for teaching microscopy (histology, cellular pathology, microbiology, and parasitology) to students studying life sciences, health sciences, and veterinary medicine.

ABBREVIATIONS

CV: Crystal violet
DPX: Dibutylphthalate polystyrene xylene
H&E: Heamatoxylin and eosin
PAS: Periodic acid schiff
RBCs: Red blood cells

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The authors declare that they have no competing interests.

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