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Connective Tissue Stains: A Review Article.

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ABSTRACT

Simple things are most commonly overlooked and some of the most common and basic parts of histopathology are stains. Stains are an integral part of routine histopathology and are commonly used in the diagnosis of various lesions and tumors. In this study we perused to collect more information on the various types of stains used to stain the different types of connective tissue components and an attempt has been made to gain more insight into knowledge, applications and also recent advances of connective tissue stains.
Keywords: Connective tissue, stains, special stains

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INTRODUCTION

Cells are the basic structural and functional units of all multicellular organisms. Tissues are aggregates or groups of cells organized to perform one or more specific functions. Epithelium is an avascular tissue composed of cells that cover the exterior body surfaces and line internal closed cavities (including the vascular system) and body tubes that communicate with the exterior (the alimentary, respiratory, and genitourinary tracts). Epithelium also forms the secretory portion (parenchyma) of glands and their ducts. In addition, specialized epithelial cells function as receptors for the special senses (smell, taste, hearing, and vision).[1]

Connective tissue comprises a diverse group of cells within a tissue-specific extracellular matrix. Connective tissue encompasses a variety of tissues with differing functional properties but with certain common characteristics that allow them to be grouped together. For convenience, they are classified in a manner that reflects this features. [1] A variety of cells with different origins and functions are present in connective tissue. Fibroblasts originate locally from undifferentiated mesenchymal cells; other cells such as mast cells, macrophages, and plasma cells originate from hematopoietic stem cells in the bone marrow, circulate in the blood, and move to connective tissue, where they remain and execute their functions. Chondrocytes synthesize and secrete the extracellular matrix, and the cells themselves are located in matrix cavities called lacunae. Adipose cells develop from mesenchymally derived lipoblasts. Bone is a specialized connective tissue composed of intercellular calcified material, the bone matrix, and three cell types: osteocytes, which are found in cavities (lacunae) within the matrix; osteoblasts, which synthesize the organic components of the matrix; and osteoclasts. Most muscle cells are of mesodermal origin, and they are differentiated mainly by a gradual process of lengthening, with simultaneous synthesis of myofibrillar proteins. [2]

An element can be broadly defined as stained, following treatment with a reagent or series of reagent, it acquires color; usually, no particles of dye are seen and the stained element is transparent. The sections as they are prepared are colorless and different components cannot be appreciated. Staining them by different colored dyes, having affinities of specific components of tissues, makes identification and study of their morphology possible. Routine stain for histological diagnosis must stain selectively not only cell nuclei and cytoplasm, but also connective tissue. Histological stain for histological diagnosis must stain killed or other non-living tissue element by methods that may appear to be specific for a particular tissue element, or group of element, but where the mechanism of staining is not understood. [3]

Physical theories states that Simple solubility e.g. fat stains are effective because the stain is more soluble in fat than in 70% alcohol. Absorption is a property by which a large body attracts to itself minute particles from a surrounding medium. Chemical theories states acid dyes stain basic elements (cytoplasm) and basic dyes stain acidophilic material (nucleus) however this far from being complete truth. Indeed hematoxylin, which is an acid dye, does not stain the cytoplasm, but (in the presence of mordant) is one of the most widely used nuclear stains. [3]

The hematoxylin and eosin stain (H&E) is the most widely used histological stain. Its popularity is based on its comparative simplicity and ability to demonstrate clearly an enormous number of different tissue structures. Hematoxylin can be prepared in numerous ways and has a widespread applicability to tissues from different sites. Essentially, the hematoxylin component stains the cell nuclei blueblack, showing good intranuclear detail, while the eosin stains cell cytoplasm and most connective tissue fibers in varying shades and intensities of pink, orange, and red. While automated staining instruments and commercially prepared hematoxylin and eosin solutions are more commonly used in today's laboratories for routine staining. [4] In a clinical histology laboratory, all specimens are initially stained with H&E and special or advanced stains are only ordered if additional information is needed to provide a more detailed analysis, for example to differentiate between two morphologically similar cancer types. [5]

Connective tissue stains are used for staining connective tissue components. The term special stains traditionally referred to any staining other than an H&E. The term "special stains" has long been used to refer to a large number of alternative staining techniques that are used when the H&E does not provide all the information the pathologist or researcher needs. It covers a wide variety of methods that may be used to visualize particular tissue structures, elements, or even microorganisms not identified by H&E staining. [5] The special stains commonly used for staining connective tissue components are Van gieson, Masson trichome, Mallory trichome, Martius scarlet blue, Phosphotungstic acid- haematoxylin stain, Periodic acid- Schiff, Aniline blue, Sirus red, Eosin etcart1.The most commonly employed method for demonstrating connective tissue is that of Van Gieson either as a counterstain with iron haematoxylin or following on the elastic fiber stains [3]. Other methods of staining use immunohistochemistry or in situ hybridization to target specific proteins or DNA/RNA sequences. These methods were sometimes also included as members of the "special stains" family. However they are quite different in method and purpose and are now typically separated into a third category known as "advanced stains". [3]

The purpose of this library dissertation is to collect more information on the various types of stains used to stain the different types of connective tissue components. So an attempt has been made to gain more insight and knowledge of the stains of connective tissue.

AIMS AND OBJECTIVES

1. To study the different stains used for staining connective tissue components and their applications in diagnosis of lesions.
2. To study the recent advances in the connective tissue staining.

CLASSIFICATION OF CONNECTIVE TISSUE STAINS

Demonstration of Collagen
- Massons trichrome stain
- Van Geisons stain

Demonstration of fibrin
- Gram Weigert
- Phosphotungstic acid hematoxylin

Demonstration of muscle striations
- Haematoxylin and eosin and trichrome methods
- [Heidenhain iron haematoxylin and Mallory's phosphotungstic acid haematoxylin]

Demonstration of elastic tissue fibres
- Verhoeff
- Orcein
- Miller's
- Weigert's resorcin-Fuchsin
- Aldehyde fuchsin

Demonstration of reticulin fibres
- Dye techniques : 1. Gordon and sweet's method
  2. Gomori's method
- Metal (silver) impregnation method

Demonstration of pigments
- Perl's Prussian blue method for ferric iron
- Lille's method for ferric and ferrous iron
- Melanin: Reducing mrthod
- Masson Fontana Stain
- Enzyme method
- Immunohistochemistry

Demonstration of carbohydrate
- Periodic Acid Schiff stain
- Alcian Blue stain
- Mucicarmine

Demonstration of Nucleic Acid stain
- Feulgen reaction
- Methyl Green pyronin stain

Demonstration of Fat
- Sudan Black stain
- Oil Red O stain

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Demonstration of Amyloid

Congo Red

Sirus red

Demonstration of Microorganism

Grams's stain

Ziehl Neelsen stain

Warthin Starry method

HISTORICAL REVIEW

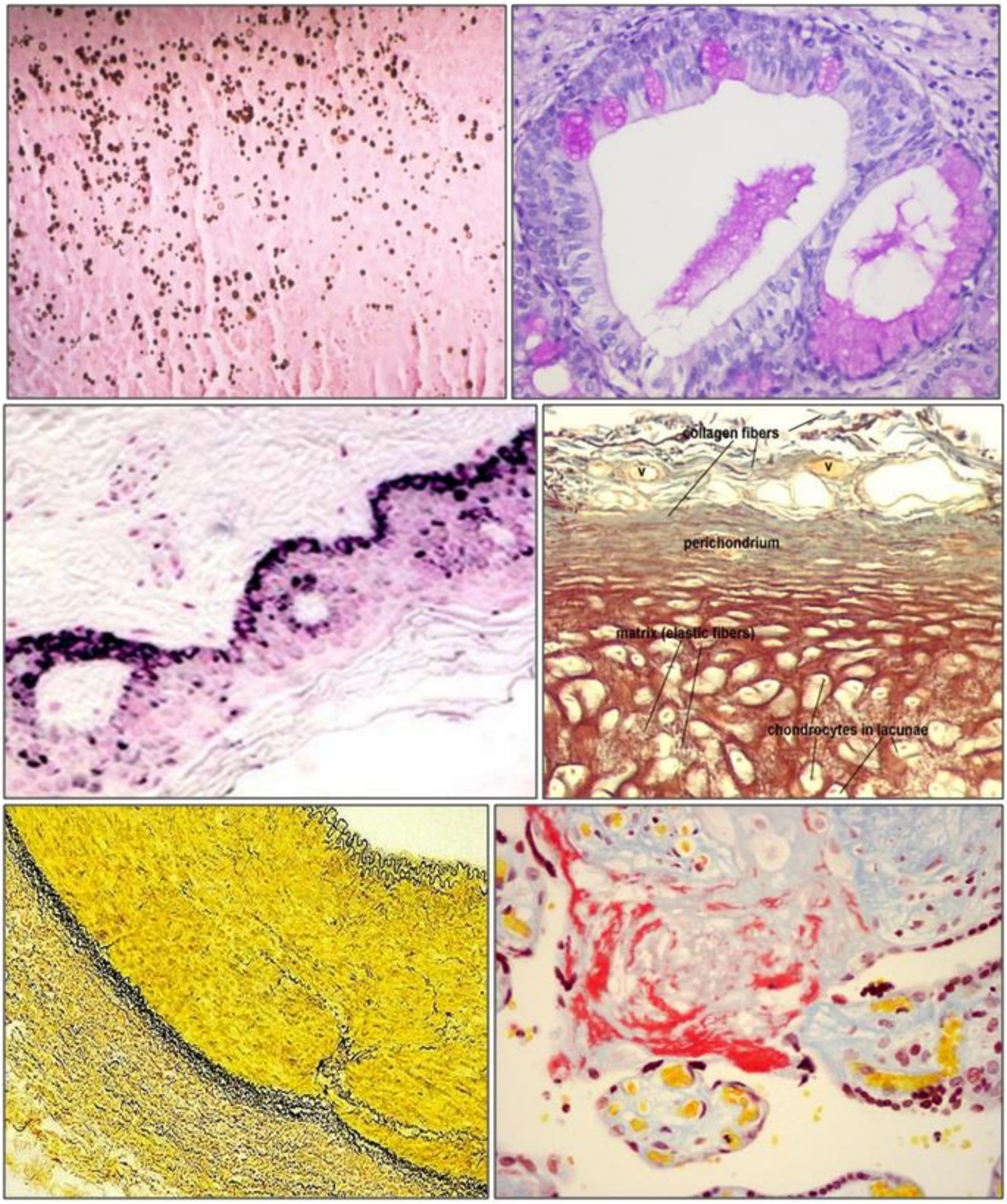
Histology is the study of the microscopic details and structures of biological cells and tissues, using light, fluorescence or electron microscopes, examining a thin slice (called a "section") of tissues, that have been previously prepared using appropriate processes called "histological techniques". [6]

The first microscope had been constructed in 1591 but had several optical problems. In 1673 Anton van Leeuwenhoek developed a simple microscope with a single lens but with improved magnification and resolution. The first microtome suitable for sectioning animal tissues was constructed in 1848. During the 19th century paraffin wax was introduced for infiltration and support during sectioning. Formalin was first used in 1893 and today is widely employed as a fixative. [6]

One of the oldest stains was Prussian blue introduced in 1774. The hematoxylin and eosin staining techniques were first described in 1875–1878 with later modifications1. Hematology took advantage from the introduction in the 1890s of Romanovsky-type staining for blood smears, including Giemsa's and May-Grunwald staining which are still fundamental in clinical practice. Louis B. Wilson was the first to develop a method using methylene dyes to stain fresh-frozen tissue of surgical specimens (1906). The periodic acid-Schiff (PAS) technique of McManus (1946) is still one of the most common diagnostic staining methods in histopathology; it superseded Best's carmine (1906) to stain polysaccharides and is widely used in liver and muscle disease. Oil Red (ORO), introduced by French in 1926 highlights the presence of fat or lipids in fresh, frozen tissue sections. [6]

Mallory's Phosphotungstic Acid Hematoxylin was first published by Mallory in 1897, this method again demonstrates another application of hematoxylin. Inthis progressive method, chemically or naturally ripened hematoxylin stains polychromatically using phosphotungsticacid as the mordant. [7]

The 1908 Verhoeff elastic method is easily the most popular elastic stain used today in histopathology laboratories. In this method, using hematoxylin, the both elastic fibers and nuclei and can be quickly stained in the laboratory. This method is often combined with the Van Gieson (1889) connective tissue stain, which gives blue–black elastic fibers, yellow cytoplasm, and red collagen [2]. The first account of a triple stain was by H. Gibbs in 1880 followed by B. W. Richardson in 1881. Animal tissues were stained with picro-carmine, iodine, malachite green, atlas scarlet and soluble blue. The triple stain became known as the trichrome stain: Tri - 3; Chrome – Color. [8]


collagen fibers
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v
perichondrium
matrix (elastic fibers)
chondrocytes in lacunae

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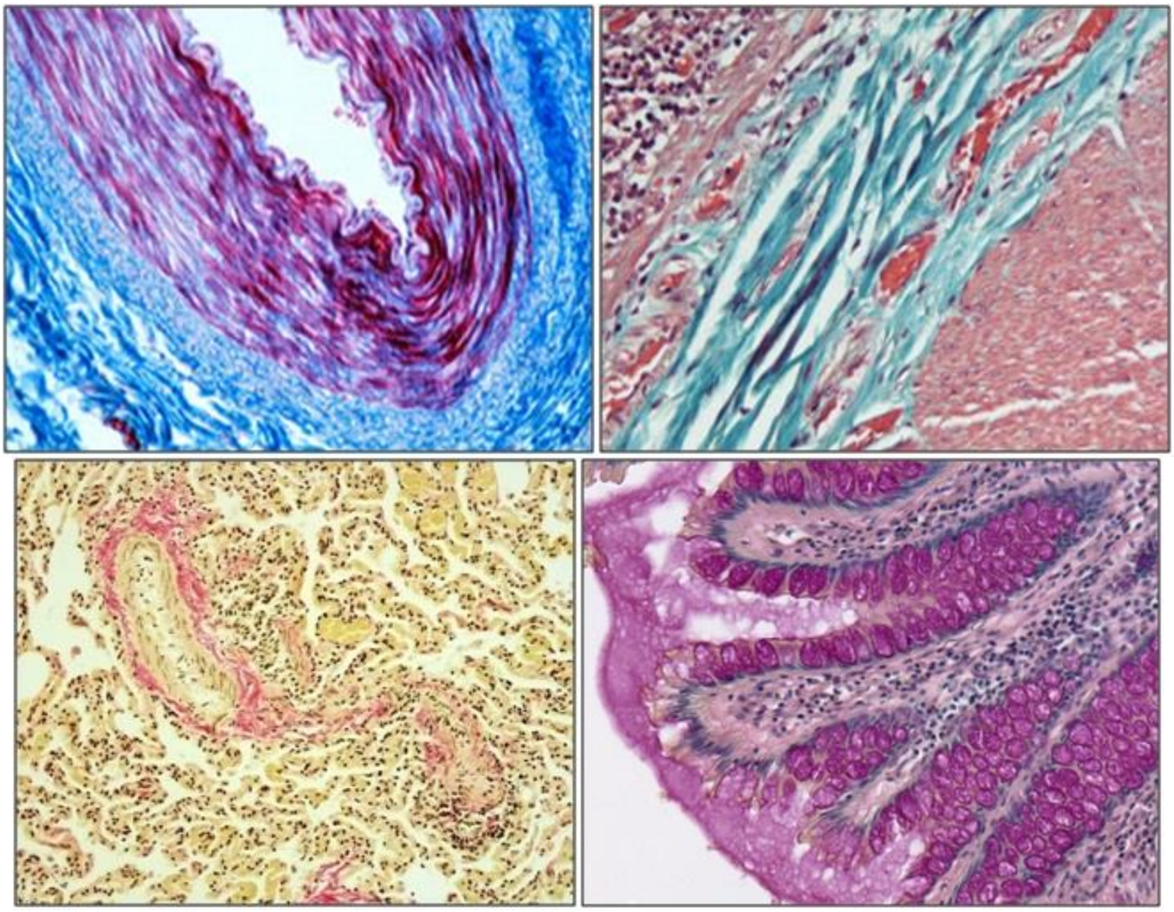

DISCUSSION

The hematoxylin and eosin stain (H&E) is the most widely used histological stain. In a clinical histology laboratory, all specimens are initially stained with H&E and special or advanced stains are only ordered if additional information is needed to provide a more detailed analysis. The term special stain traditionally refers to any staining other than an H&E. The term "special stains" has long been used to refer to a large number of alternative staining techniques that are used when the H&E does not provide all the information to the pathologist or the researcher. It covers a wide variety of methods that can be used to visualize particular tissue structures, elements, or even microorganisms not identified by H&E staining.

The special stains commonly used for staining connective tissue components are Van gieson, PAS, Masson trichome, Mallory trichome, Martius scarlet blue, Phosphotungstic acid- haematoxylin stain, Sirus red, Eosin, Verhoff- Van Gieson stain, Masson Fontana stain, Von kossa to name a few.

The trichrome stain is one of the most utilized special stains in the histopathology Laboratory. As the name implies the modified MT staining involves three colors of staining dyes. MT staining is able to differentiate clearly the important morphological keys such as keratin, haemoglobin, and muscle fiber (red colour), cytoplasm and adipose cells (light red or pink), cell nuclei (dark brown to black) and collagen fiber which are stained blue. Clear differentiation of morphological and anatomical structure in the stained tissue are advantageous and provide further understanding of histopathological study in future. The MT staining is widely used in medical pathology laboratories to differentiate between collagen and smooth muscle in tumors, determine the increase of collagen in disease such as cirrhosis and it is also a routine stain for liver and kidney. [16]

Van Gieson's Stain is a mixture of picric acid and acid fuchsin. It was introduced to histology by American neuropsychiatrist and pathologist Ira Van Gieson. It is the simplest method of differential staining of collagen and other connective tissue. Staining methods such as Van Gieson's and the various forms of trichrome have been regarded as specific for collagen and, despite their documented limitations, are still being used. Both of these methods fail to reveal very thin collagen fibers, a disadvantage which can, under certain circumstances, lead to a substantial underestimation of collagen content. Perhaps more surprising is the continued use of hematoxylin and eosin to detect collagen, even though eosin has no specificity for collagen. Sirius red F3BA dissolved in a saturated picric acid solution consistently stains thin collagen fibers, do not fade, and is suitable for use with polarized light microscopy. [39]

Martius scarlet blue (MSB) is a modifications to the Masson technique to enable older deposits of fibrin to be demonstrated. The standard MSB technique employs Martius yellow (acid yellow), brilliant crystal scarlet (acid red), and soluble blue (methyl blue) (acid blue). The main advantage is that early fibrin deposits may be stained by this dye. [4] Orange G, Naphthalene Blue Black CS, Chicago Red are also used to stain fibrin.

Various methods for staining elastic tissue exist such as Weigert's, Orcein, Gomori and Verhoeff. The main challenge in staining is to differentiate elastic fibers from collagen and smooth muscle in various tissues. This is not possible by use of just one stain, and a combination of stains is required. Van Geison stain is a commonly used counterstain, which when combined with primary elastic stains helps in better differentiation of elastic from collagen and smooth muscle. Frederick Herman Verhoeff, an American surgeon and pathologist, then modified Van Gieson stain in 1908, as a method to differentiate collagen and other connective tissues, and highlight elastic fibers in particular. VVG is a two-part combination stain that enables differentiation of some connective tissue components in a tissue which are not easily distinguished by H&E staining. Elastic Verhoeff-Van Gieson staining is easily performed, cheap and available in almost every histopathology laboratory. It finds usefulness in a wide array of cutaneous disorders ranging from inflammatory to neoplastic conditions. It has the potential to narrow the differential diagnosis of inflammatory disorders as well as neoplastic processes and used to evaluate tumor thickness in melanomas that arise in nevi. [19]

Papanicolaou stain is a multichromatic staining cytological technique developed by George Papanikolaou, the father of cytopathology. PAP staining is used to differentiate cells in smear preparations of various bodily secretion. It is used to differentiate in medical diagnosis suspected cells types in samples for cytological cancer, e.g. cervical cancer. It is used for the initial evaluation to differentiate nuclei,cytoplama and squamous cells and examined under microsope.

Periodic acid–Schiff (PAS) is a staining method used to detect polysaccharides such as glycogen, and mucosubstances such asglycoproteins, glycolipids and mucins in tissues. PAS staining is mainly used for staining structures containing a high proportion of carbohydrate macromolecules (glycogen, glycoprotein, proteoglycans), typically found in e.g. connective tissues, mucus, the glycocalyx, and basal laminae.

The Fontana-Masson silver (FMS) stain is a histochemicaltechnique that oxidizes melanin and melanin-like pigments as it reduces silver. The stain is relatively specific for infections caused by Cryptococcus neoformans.[40] Excessive amounts of melanin pigments may hamper histopathologic assessments of melanocytic lesions by obscuring cellular morphology.

Three main techniques are currently employed for the histological demonstration of amyloid: 'metachromasia' with triphenylmethane dyes (eg, crystal violet); staining with substantive cotton dyes (Congo red, Sirius red), with or without fluorescence or polarization microscopy; and fluorescence with thiazole dyes (eg, thioflavine-T). [41] Congo Red is the most widely used stain for Amyloid at the microscopic level.

Sudan Black and Oil Red O stains are used for demonstration of fats. Sudan black B was introduced as a specific fat stain for the detection of lipids in tissue sections by Lison (1934). Burdon (1946) modified the procedure for demonstrating the intracellular fatty materials in bacteria by preparing dried fixed films for lipid staining with Sudan black B. [42]

The advantages of special stains are their easy handling properties. These stains are economical, can be easily prepared in the laboratory and do not require special expertise for preparation. These stains are beneficial in demonstrating the specific characteristics of different tissues.

There certain limitations in connective tissue staining. Van gieson stain and Masson Trichrome stains fail to reveal very thin collagen fibers, a disadvantage which can, under certain circumstances, lead to a substantial underestimation of collagen content. [39] In Van gieson staining, there is tendency for the red color to fade, whatever mounting medium is used. It is challenging to differentiate elastic fibers from collagen and smooth muscle in various tissues and is not possible by use of just one stain such as Verhoeff stain, and a combination of stains is required. Orcein staining potential diminish after 4 days and produces less intense color than Verhoff's stain. Weigert's Resorchin- Fuschin stain is a time consuming technique. Gordon and Sweet's method gives much less background and nuclear staining. Fat staining is relatively technique sensitive, Some neutral fat may be lost during staining, so mounting should be done carefully.

Sirius red F3BA dissolved in a saturated picric acid solution consistently stains thin collagen fibers, do not fade, and is suitable for use with polarized light microscopy. To avoid the fading of red color in Van gieson, Curtis (1905) suggested the use of ponceau S as a substitute for acid fuchsin but this dye, unfortunately, stains young collagen fibres less well than do acid fuchsin. In elastic staining a combination of elastic stains and Van gieson counterstain is used to differentiate elastic fibers from collagen and smooth muscle in various tissues. When poor results are obtained with Gordon and Sweet's method the stain should be repeated with particular attention to the diamine silver solution. [4]

A rapid technique is proposed using Oil Red O stain with hematoxylin as a nucleic counterstain, which considerably reduces the delay, relative to usual staining procedures. Oil Red O and Harris' hematoxylin are used in the 15 min procedure. Coloration is excellent and distortion minimal. [43]

Staining with special stains, we should keep in mind the following considerations - Special staining often requires the use of unusual stains and reagents that are available from only a few sources. Special staining requires broad knowledge of the tissue or cells targeted. Staining with special stains, we should take care to collect, fix and prepare the specimen in a manner that will maintain the molecule of interest within cells or tissues. IHC is a highly reproducible and reliable assay when an IHC test is run on adequately fixed and processed paraffin-embedded tissue under the identical staining procedure as that of the antibody validation process. Now IHC tests are standardized from preanalytic, analytic, to postanalytic phases. [44] IHC has been adapted to the identification and demonstration of both prognostic and predictive markers, with corresponding requirements for semi-quantitative reporting of results. The widespread use of IHC and the demands for comparison of qualitative and semi-quantitative findings among an increasing number of laboratories have resulted in a growing focus on method reproducibility and have led to a new emphasis upon standardization. The development of the hybridoma technique facilitated the development of IHC and the manufacture of abundant, highly specific monoclonal antibodies, many of which found early application in staining of tissues.

CONCLUSION

Connective tissue comprises a diverse group of cells within a tissue-specific extracellular matrix. A variety of cells with different origins and functions are present in connective tissue. The hematoxylin and eosin stain (H&E) is the most widely used histological stain. Special or advanced stains are only planned if additional information is needed to provide a more thorough analysis and it covers a wide variety of methods that can be used to visualize particular tissue structures, elements, or even microorganisms not identified by H&E staining. There are various special stains which are used to study the different connective tissue components.

Connective tissue stains have been used extensively for diagnosis of tumors of various origins. Understanding these techniques not only aids us in performing of our staining procedures effectively but also can facilitate the innovation of new methods.

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