***Review Article***

# A review of basic histopathological staining techniques

**Abstract:**

Tissue staining techniques have been an integral part of pathology for over a century. These techniques allow pathologists to visualize the structure and composition of tissues under a microscope. These techniques play a crucial role in the diagnosis and treatment of a wide range of diseases and disorders.

The most commonly used staining techniques include hematoxylin and eosin (H&E) staining, immunohistochemistry (IHC), and special stains. H&E staining is a routine staining method that is used to identify the cellular and tissue structures. The hematoxylin stains the nuclei blue, and the eosin stains the cytoplasm and extracellular matrix pink.

IHC staining utilizes antibodies to target specific proteins in tissues, providing information about the tissue's molecular composition. This technique is used to diagnose and classify cancers, identify infectious agents, and assess the tissue's immune response.

Special stains are used to highlight specific structures or substances within tissues, such as lipids, carbohydrates, and minerals. These stains are used to identify particular cells, such as bacteria or fungi, and help to diagnose diseases such as tuberculosis and fungal infections.

Key words: Tissue Staining, Technique, Slides, Pathology slides, Tissue slides, Stains, Hematoxylin and Eosin

## Introduction

Access to diagnostic services, including pathology, is essential for the prevention and treatment of diseases, particularly in some poor resource and underserved communities. However, these communities often face barriers to accessing these services, including limited funding and inadequate healthcare infrastructure. Tissue staining techniques can play an essential role in addressing these challenges by providing low-cost and effective diagnostic solutions.

In resource-poor settings, the use of basic staining techniques, such as H&E staining, can be an effective tool for diagnosing many diseases. These techniques are relatively inexpensive and require only basic laboratory equipment, making them ideal for use in low-resource settings. Additionally, the use of rapid diagnostic tests, such as immunohistochemistry, can provide a valuable and accurate diagnoses, enhancing the need diagnostic accuracy in laboratory tests[1-4].

However, the availability of reagents, equipment, and trained personnel remains a significant challenge in underserved communities. Strategies such as point-of-care testing, telepathology, and training local personnel can help to address these challenges. Point-of-care testing can provide on-site diagnoses, reducing the need for transport of samples to central laboratories. Telepathology, where images of stained tissues are shared remotely for diagnosis, can help connect underserved communities with expert pathologists in more resourced settings. Training local personnel, such as medical laboratory technicians, can help build local capacity and improve the quality of diagnostic services in underserved areas.

Tissue staining techniques are methods used to enhance the contrast between the different components of a tissue sample, making it easier to visualize and study the individual cells and structures[1-4]. This is achieved using dyes or stains that selectively bind to certain cellular components, highlighting their morphology, distribution, and function. Tissue staining techniques play a critical role in histology, pathology, and biomedical research by allowing researchers and physicians to identify and study the structure and function of different tissues and organs at the cellular level. Tissue staining techniques are critical tools in pathology for diagnosing, characterizing, and understanding various diseases and conditions.[1] Tissue staining techniques are essential tools in pathology that help pathologists diagnose and understand diseases, guide treatment decisions, and advance biomedical research. Here are some key ways tissue staining techniques are important in pathology. First and foremost is identifying cellular and tissue abnormalities. Tissue staining techniques help pathologists visualize and identify abnormal cells, tissues, and structures in tissue samples[2-8]. This is important for diagnosing diseases such as cancer, infectious diseases, and autoimmune disorders[2-8]. Secondly, characterizing the disease processes; By staining tissues with specific dyes, pathologists can determine the type, location, and extent of disease processes in the body. This helps guide treatment decisions and monitor the progression of the disease. Thirdly, determining treatment options; certain staining techniques, such as immunohistochemistry, can help identify specific proteins or molecular markers present in diseased tissues. This information can guide treatment decisions and develop targeted therapies. Lastly, research and discovery. Tissue staining techniques are also essential for research in pathology and biomedical sciences. Therefore, by staining tissues and analyzing the resulting images, researchers can study the structure, function, and behavior of cells and tissues in health and disease.[2] The purpose of a review paper on tissue staining techniques would be to explore the different methods and applications of tissue staining in biomedical research and pathology. The paper could provide an overview of the various staining techniques and dyes used and their advantages and limitations. It could also discuss the different applications of tissue staining, including diagnosing and characterizing diseases, identifying specific biomarkers, and studying cellular processes and interactions. The review paper could also delve into the challenges and limitations of tissue staining, such as the potential for artifacts and the need to interpret results carefully. Additionally, it could highlight recent advances and innovations in tissue staining, such as developing new dyes and techniques and their potential impact on biomedical research and clinical practice. Overall, this review of tissue staining techniques could provide valuable insights into this critical aspect of pathology and biomedical research and highlight its importance for advancing our understanding of health and disease.

**Types of Tissue Staining Techniques**

**Hematoxylin and Eosin (H&E) Staining**

This is the most widely used staining technique in histology. It is a simple and versatile technique that allows pathologists to identify and characterize tissues based on the staining properties of different cellular components. [3,8-16] The H&E staining process involves two main dyes: hematoxylin and eosin. The tissue sample is first fixed, dehydrated, and embedded in paraffin wax, which preserves the tissue structure and allows it to be sectioned into thin slices. The tissue sections are then deparaffinized and rehydrated before being stained with hematoxylin and eosin. Under the microscope, the stained tissue sections show various colors and patterns that reflect cells and tissues' different types and arrangements. For example, nuclei appear blue-purple, while cytoplasm appears pink-red. Connective tissue fibers and extracellular matrix components may appear in shades of pink or blue depending on their composition. H&E staining is widely used for diagnostic purposes in pathology, as it allows pathologists to visualize and identify various tissue structures and abnormalities.[3] Therefore, it is used for research, providing a detailed and easily interpretable view of tissue morphology and structure.

**Staining Process**

The H&E staining process involves a series of steps that are typically performed on tissue sections mounted on glass slides. The basic steps involved in the staining process are as follows: The first step is fixation, which involves fixing the tissue sample in a suitable fixative, such as formalin or paraformaldehyde, to preserve its morphology and prevent autolysis or degradation. The tissues are then dehydrated in a series of graded alcohols, typically starting with low concentration (e.g., 70% ethanol) and gradually increasing to higher concentration (e.g., 95% and 100% ethanol). This step removes any water from the tissue, allowing it to be permeable to the staining reagents. [3] The tissue is then cleared with a suitable solvent, such as xylene or limonene, to remove any remaining alcohol and to make the tissue transparent. The tissue is then infiltrated with a suitable embedding medium, such as paraffin wax or resin, which supports and helps preserve the tissue morphology. The tissue sections are mounted onto glass slides, typically coated with a suitable adhesive such as poly-L-lysine, and allowed to dry. The tissue sections are then subjected to the staining process, which typically involves the following steps. Deparaffinization, the slides are immersed in xylene or a xylene substitute to remove the paraffin wax and make the tissue accessible to the staining reagents. The slides are then rehydrated by passing them through a series of graded alcohols, typically in reverse order to that used during dehydration. Staining, the slides are then stained with hematoxylin, which is a basic dye that stains the nuclei blue-purple, followed by eosin, which is an acidic dye that stains the cytoplasm and extracellular matrix pink to red.

Dehydration and clearing, the slides are then dehydrated and cleared in a series of graded alcohols and xylene to remove excess stains and make the tissue transparent. Mounting, the slides are then mounted with a suitable mounting medium, typically a synthetic resin, and covered with a coverslip to protect the tissue and prevent the mounting medium from drying out. The stained tissue sections can then be examined under a light microscope, allowing for the visualization and identification of different cellular and tissue components.

**Applications of H&E Staining**

H&E staining is a widely used staining technique in histology and pathology as it is relatively simple, inexpensive, and provides high contrast and resolution, allowing for the identification and diagnosis of a wide range of diseases and conditions.[8,16] It is used to identify and differentiate various types of tissue, including normal and abnormal tissue, and to evaluate the presence and severity of cellular and tissue abnormalities, such as inflammation, infection, and cancer.[5] Some of the main applications of H&E staining include the diagnosis of diseases. H&E staining is commonly used in the diagnosis of various diseases and conditions, including cancer, infections, inflammation, and autoimmune disorders. The staining allows pathologists to identify and differentiate different cell types and tissue structures and to evaluate the presence and severity of cellular and tissue abnormalities.

Identification of tissue structures: H&E staining identifies and differentiates different tissue structures, including epithelial, connective, muscular, and nervous tissues. This is particularly useful in research studies that aim to understand the structure and function of different tissues. Evaluation of treatment efficacy. H&E staining can be used to evaluate the efficacy of treatment regimens for various diseases, including cancer. By examining tissue samples before and after treatment, pathologists can assess the impact of the treatment on the tissue and identify any residual disease. H&E staining is an important tool in teaching and education in histology and pathology, as it allows students and trainees to visualize and understand the different cellular and tissue components. In addition, *H*&E staining is used extensively in research studies to investigate the structure and function of tissues and to identify and characterize different cell types. It is particularly useful in studies that aim to understand the pathogenesis of diseases and to develop new diagnostic and therapeutic approaches[5, 16].

**Advantages and Limitations of H&E Staining**

**Advantages of H&E staining**. Easy to perform: H&E staining is a relatively simple and straightforward staining technique and does not require specialized equipment or extensive training. Widely available: H&E staining is widely available in most histology and pathology laboratories, making it a convenient and accessible staining technique for researchers and clinicians. High contrast. H&E staining provides high contrast between different cellular and tissue components, making identifying and differentiating different structures and cells easy. Cost-effective. H&E staining is a cost-effective staining technique, as it requires only basic staining reagents and materials. Compatible with other techniques. H&E staining can be combined with other techniques, such as immunohistochemistry, to provide additional information about tissue components and structures[5,8,16].

**Limitations of H&E staining**. H&E staining provides limited specificity for different cellular and tissue components, as the staining is based on the general properties of different cells and tissues rather than specific molecular markers. H&E staining provides information about cellular and tissue morphology but does not provide information about the cellular function or molecular interactions. H&E staining may not be sensitive enough to detect certain cellular or tissue abnormalities, particularly in cases where the abnormality is subtle or localized. H&E staining may introduce artefacts, such as folding or distortion of tissue sections, which can affect the interpretation of the staining results. The interpretation of H&E staining results can be subjective and may vary depending on the pathologist's or researcher's experience and expertise [5, 8,16].

**Special Stains**

These are special procedures that highlights particular constituents of cells that would aid in arrival of a definite diagnosis. Prussian Blue technique (Figure 1) is commonly used to demonstrate the presence of iron salts in tissue[17,18]. Reticulin can be highlighted to confirm or refute distortion in microarchitecture (Figure 2). Special stains can also be used in histopathology to confirm the presence of some organisms, for example Giemsa staining technique (figure 3) can highlight the *helicobacter pylori* rods in gastric antrum, helping the pathologist to render a diagnosis of H. Pylori gastritis. In similar vein, viral inclusion bodies can be demonstrated in liver using Shikata Orcein technique[19] that gives a brown or purple appearance to HBsAg inclusions and stains copper associated proteins as dark purple (Figure 4).

**Ziehl-Neelsen Stain.** The Ziehl-Neelsen stain is a special type of staining method utilized to detect acid-fast bacteria, particularly Mycobacterium tuberculosis, the causative agent of tuberculosis. It was first developed by the German bacteriologists Franz Ziehl and Friedrich Neelsen in 1882. The Ziehl-Neelsen staining process involves several steps. First, a heat-fixed smear of the clinical sample is covered with a primary stain, usually carbol fuchsin, and heated gently to allow the stain to penetrate the cell walls of the acid-fast bacteria. The slide is then washed with acid alcohol, which removes the stain from non-acid-fast bacteria and other tissue components.[11,20-22] The slide is then counterstained with a contrasting color, usually methylene blue, which stains the non-acid-fast bacteria and other tissue components blue, while the acid-fast bacteria retain their pink or red color. Under the microscope, non-acid-fast bacteria and other tissue components appear blue, while acid-fast bacteria appear pink or red. The Ziehl-Neelsen stain is a specific and sensitive method for detecting acid-fast bacteria, particularly Mycobacterium tuberculosis, in clinical samples.

The Ziehl-Neelsen stain is widely used in clinical microbiology and is considered the gold standard for diagnosing tuberculosis. It is also used to diagnose other acid-fast bacterial infections, such as leprosy and nocardiosis. In addition, the Ziehl-Neelsen stain can be useful in identifying acid-fast bacteria in environmental samples, such as soil and water. One limitation of the Ziehl-Neelsen stain is that it requires careful preparation and interpretation, as other acid-fast bacteria and certain tissue components may stain pink or red and could be mistaken for Mycobacterium tuberculosis. Nevertheless, the Ziehl-Neelsen stain remains a valuable tool in clinical microbiology, allowing clinicians and researchers to rapidly and accurately diagnose acid-fast bacterial infections.

**Ziehl-Neelsen Staining Process.** The procedure starts with the bacterial sample being heated or chemically fixed on a microscope slide. The mycolic acid in the bacterial cell wall is next stained with carbolfuchsin, a red dye containing phenol, which is poured across the slide. These samples are now prepped properly. The slide is then heated to allow the dye to enter the bacterial cell wall more easily. The dye from the non-acid-fast bacteria comes off when the slide is washed with acid-alcohol. However, the dye in the acid-fast bacteria persists after the alcohol or acetone wash. This method uses methylene blue as a counterstain to highlight the contrast between the red-stained acid-fast bacteria and the blue-stained non-acid-fast bacteria on the slide[11,20-22].

**Applications of Ziehl-Neelsen Staining.** The most common application of the Ziehl-Neelsen staining is the classification and further of the genus Mycobacterium.[12] The area is important because tuberculosis has ravaged mankind for centuries, and there is a need for a lasting solution. It is also applied to positively identify Cryptosporidium and certain fungal species as well as acid-fast or non-acid-fast bacteria.

**Advantages of Ziehl-Neelsen Staining.** It is a relatively inexpensive and modest technique despite its huge accuracy and precision in the identification of these bacteria. It is its central benefit and strength, which is further complemented by its very wide application. In acid-fast bacteria detection, it remains the global leader in accuracy and precision[11,20-22].

**Limitations of Ziehl-Neelsen Staining.** It has very low sensitivity and specificity to detect acid-fast bacteria in certain regions. For instance, extrapulmonary samples show huge failures.[12] It requires extremely well-trained expertise and specialized equipment to achieve optimal results. Likewise, it suffers from the same problem as its related acid tests: the failure to distinguish between living and non-living bacteria[23].

**Periodic Acid-Schiff (PAS) Stain.** This technique is particularly useful for identifying and localizing glycogen, basement membranes, and fungal organisms in tissue specimens.[7] The PAS staining process involves several steps. First, the tissue sample is fixed, embedded, and sectioned into thin slices. The sections are then treated with periodic acid, which oxidizes the carbohydrates in the tissue and creates aldehydes. Glycogen appears as magenta or red granules within cells, while basement membranes appear as a bright magenta line around cells and tissues.[7,24] Depending on their composition and staining properties, fungal organisms may appear magenta or red.

The PAS stain is particularly useful in diagnosing and characterizing various diseases, including glycogen storage diseases, certain renal diseases, and some fungal infections. The technique can also be used in research to investigate the structure and function of carbohydrates and glycoproteins in tissues and cells.[7] One limitation of PAS staining is that it is not specific to particular types of carbohydrates or glycoproteins and can stain a wide range of substances. This can sometimes make interpreting the results more challenging, especially in cases with overlapping or background staining. Nevertheless, PAS staining remains a valuable tool in histology and pathology, allowing researchers and clinicians to gain insights into the molecular and cellular processes underlying various diseases and conditions[7,24-26].

**Periodic Acid-Schiff (PAS) Staining Process.** The PAS process is simple and follows a series of straightforward steps. The tissue sample is fixed and embedded in paraffin before small parts of the sample are chopped and placed on a slide. These slides are then deparaffinized, and the sample is hydrated with decreasing concentrations of alcohol baths and distilled water. The alcohol baths can be 100%, 80%, and so forth. After that, the slides are covered with Schiff's reagent for approximately half an hour. Then, cold water from the faucet is run over them for 5-10 minutes. After reacting with the aldehydes, a pink or magenta color is created. The last step after the magenta colors is washing slides and counterstaining with hematoxylin, which shows up as blue in the cell nuclei.

**Application of Periodic Acid-Schiff (PAS) Staining.** The application of this laboratory technique is broadly categorized as the diagnosis of glycogen storage diseases and the diagnosis of certain cancers. PAS staining is a crucial diagnostic tool for detecting glycogen accumulation in tissues, which is a hallmark of glycogen storage diseases.[7] In cancer diagnosis, PAS is applied because certain malignancies, like lymphoma and melanoma, produce a lot of mucopolysaccharides. It can help identify some of these cancers. Lastly, the lesser-known application is detecting or identifying microorganisms like fungi and parasites. These organisms are known to store enormous amounts of glycogen and other carbohydrates, which makes PAS staining perfect for detecting them.

**Advantages of Periodic Acid-Schiff (PAS) Staining.** PAS staining is highly sensitive and can respond to minute amounts of carbohydrates in tissues and samples. It makes it very useful in healthcare facilities that utilize it for various functionalities. Additionally, it is highly compatible with other staining techniques. Therefore, it is commonly combined with numerous other techniques to provide many optimal results.[7] For instance, immunohistochemistry is common when searching for additional cell morphology information. It is why PAS is considered the most versatile staining. It can be applied to a wide range of carbohydrates such as glycogen, mucopolysaccharides, and mucin and carbohydrate-rich compounds such as glycolipids, certain glycoproteins, and certain fungi.[7,24-26.]

**Limitations of Periodic Acid-Schiff (PAS) Staining.** The test offers very nonspecific staining and thus is very difficult to understand the difference in detected carbohydrates. This flaw is coupled with its extremely poor time management. The results take extremely long to manifest, in some instances even days to provide results and thus making it relatively inefficient because time is always ticking in hospitals. Also, PAS has a limited specificity in certain cases and can create false detections.[7] For instance, formalin pigment can react with periodic acid and Schiff's reagent, giving false results. Such cases show the frailties of the technique and how despite its huge benefits, it still can be damaging[28].

**Trichrome Stain.** Trichrome staining is a histochemical technique for visualizing collagen and other connective tissue fibers in tissue samples.[8] The technique is particularly useful for identifying and quantifying the amount and distribution of collagen in tissues. The trichrome staining process involves several steps. First, the tissue sample is fixed, embedded, and sectioned into thin slices. The sections are then treated with a mixture of three dyes: hematoxylin, which stains nuclei blue; acid fuchsin or fast green, which stains cytoplasm and muscle fibers red or green, respectively; and a trichrome stain, which stains collagen and other connective tissue fibers blue or green. Under the microscope, the stained tissue sections show blue, red, or green areas, and blue or green, indicating the presence of nuclei, cytoplasm, and connective tissue fibers, respectively. The intensity and distribution of the blue or green color can be used to quantitatively assess the amount and organization of collagen in the tissue[29-31].

Trichrome staining is particularly useful in diagnosing and managing various diseases, including fibrosis and other connective tissue disorders. It can also be used in research settings to investigate the structure and function of collagen and other connective tissue fibers in tissues and cells. One limitation of trichrome staining is that it can be time-consuming and require specialized equipment and expertise. Interpreting the results can also be challenging, as collagen staining can vary depending on tissue fixation and processing factors. Nevertheless, trichrome staining remains a valuable tool in histology and pathology, allowing researchers and clinicians to gain insights into the molecular and cellular processes underlying various diseases and conditions.[8] A good example of a trichrome stain is the Masson Trichrome staining technique

**Masson's Trichrome Stain.** This technique is used to differentiate collagen fibers (blue), muscle fibers (red), and other tissues (green) in tissue samples. This technique was first described by Raymond Masson in 1929. The Masson trichrome staining process involves several steps. First, the tissue sample is fixed, embedded, and sectioned into thin slices.[10] The sections are then stained with a mixture of three dyes: Weigert's iron hematoxylin, which stains nuclei blue; Biebrich scarlet-acid fuchsin, which stains cytoplasm and muscle fibers red; and a trichrome stain, which stains collagen and other connective tissue fibers blue or green. Under the microscope, the stained tissue sections show blue, red, and green areas, indicating nuclei, cytoplasm and muscle fibers, and connective tissue fibers, respectively. The intensity and distribution of the blue or green color can be used to assess the amount and organization of collagen in the tissue.[10,29-31]

Masson's trichrome staining is particularly useful in identifying and quantifying fibrosis and other connective tissue disorders. It is also useful in distinguishing between muscle and collagen fibers in tissue samples, making it useful in the diagnosis of muscle diseases. One limitation of Masson's trichrome staining is that it can be time-consuming and require specialized equipment and expertise. Interpretation of the results can also be challenging, as collagen staining can vary depending on factors such as tissue fixation and processing.[10] Nevertheless, Masson's trichrome staining remains a valuable tool in histology and pathology, allowing researchers and clinicians to gain insights into the molecular and cellular processes underlying various diseases and conditions.

**Trichrome Staining Process.** The steps involved in the trichrome staining process are easy to follow despite the expertise or experience needed. In the first stage, the tissue sample to be tested is fixed and embedded in paraffin before being chopped, and some of these sections are placed on slides. These tissue samples on slides are deparaffinized and rehydrated in distilled water to provide the perfect environment for the next stage. The samples are then stained with the available or targeted dye combinations. Most commonly, the dye combination may feature hematoxylin, which stains the nuclei blue; acid fuchsin, which stains collagen fibers and muscle fibers red; and light green or yellowish light green SF, which stains the cytoplasm and other structures green. Lastly, the slides are washed and dehydrated in concentrated alcohol solutions and cleared with xylene.

**Applications of Trichrome Staining.** Trichrome staining is primarily applied in diagnosing connective tissue disorders and gastrointestinal disorders and diagnosing gastrointestinal disorders to a smaller extent. Conditions such as scleroderma and fibrosis can be detected and diagnosed by staining and identifying abnormal collagen fibers in tissue samples.[7] Similarly, the method can easily diagnose gastrointestinal disorders, such as cryptosporidiosis, giardiasis, and microsporidiosis.[7] The tests stain and identify alien organisms which might cause diseases. The last application which has not been advanced significantly is muscular complications diagnosis. Trichrome staining can be applied in muscle complications such as muscular dystrophy. Doctors can determine these conditions by staining and identifying abnormal collagen fibers in tissue samples.

**Advantages of Trichrome Staining.** The process yields very high-contrast images, which helps with diagnoses of various conditions explained above. The identification of alien cells and structures is significantly made easier by these high-contrast images. More importantly, it is applicable in a broad spectrum. However, the results can last long, giving the method ubiquitous application. After the procedure, the slides can be stored for extremely extended periods of time without any major change or loss in staining density.[7, 29-31]

**Limitations of Trichrome Staining.** The staining procedure is extremely time-consuming, with results averaging more than a day and a week in extreme cases. It is a huge flaw that has devastated the application of the trick on a large scale. It also suffers from non-specificity in staining, a common drawback of most staining techniques. Its results sometimes lack the specific nature that can help distinguish between various tissues and components.[7] Lastly, it is a fairly complex technique requiring the utmost precision and expertise to pull off the most optimal results. Therefore, its application is sometimes limited by its huge technical demands[29-31].

**Gram Stain.** The Gram stain is a differential staining technique used to classify bacteria based on the structure of their cell walls. This technique is named after the Danish bacteriologist Hans Christian Gram who developed it in 1884. The Gram staining process involves several steps. First, a smear of the bacterial culture is fixed onto a glass slide and then flooded with crystal violet. This primary stain binds to the peptidoglycan layer of bacterial cell walls.[9] The slide is then washed with a decolorizing agent, such as alcohol or acetone, which can remove the crystal violet from some bacteria. The slide is then stained with a counterstain, usually safranin or fuchsin, which stains the decolorized bacteria a contrasting color. Under the microscope, Gram-positive bacteria appear purple or blue, while Gram-negative bacteria appear pink or red. Gram-positive bacteria have a thick peptidoglycan layer in their cell walls, which retains the crystal violet stain after decolorization.

In contrast, Gram-negative bacteria have a thin peptidoglycan layer and an outer membrane that is not stained by crystal violet and can be decolorized by the decolorizing agent. The Gram stain is a widely used and simple technique for rapidly identifying and classifying bacteria in clinical and research settings. It is particularly useful in identifying the causative agent of bacterial infections, as different bacterial species often require different treatment strategies.[9] The Gram stain can also provide valuable information about bacterial cells' morphology, arrangement, and structure. One limitation of the Gram stain is that it is not always reliable or definitive. Some bacteria, such as those with atypical cell wall structures or those that antibiotics or other factors have damaged, may not stain or stain inconsistently. Nevertheless, the

Gram stain remains a fundamental and widely used technique in microbiology and infectious disease diagnosis.

**Gram Staining**

The gram stain is a common laboratory test that checks for bacteria at any suspected infection and can be done on certain body fluids. It helps identify and differentiate bacteria species because of the different cell wall compositions.[9] The test yields gram-positive and gram-negative results, two broad classifications based on color sets. The gram-positive organisms appear blue to purple under gram stain due to thick layers of peptidoglycan.[9] In contrast, gram-negative organisms have very thin walls of peptidoglycan and, under the gram stain, appear red to pink.[9,32-34] However, the test is not absolute as some bacteria are gram-variable and can stain irregularly.

**The Gram Staining Process.** The major steps involved in the test are collecting, processing, and staining the sample. The collection involves taking a swab of a sample from the site. Samples include stool, urine, sputum, pericardial, pleural, and cerebrospinal fluids. Processing involves smearing samples on glass slides and applying stains in 4 steps. Applying primary stain, adding mordant, rapid decolorization with acetone, and counterstaining with safranin. The last stage is an examination of the smears to see the present bacteria, whether gram-positive or gram-negative.

**Applications of Gram Staining.** The major application is the identification of bacteria with its swift and reliable capabilities. Gram stain identifies bacterial species and determines their wall composition to help in placing them in the correct taxonomy.[9] Additionally, Gram stain is applied to diagnose bacterial infections in clinical microbiology. Common conditions include UTI and pneumonia, among many others. Lastly, it is used as a quality control in microbiology. It, therefore, helps monitor the purity of bacteria cultures and the accuracy of microbiological tests.

**Advantages of Gram Staining.** The process requires the most minimal resources to complete due to its simple and straightforward procedure. It is thus not costly or resource-intensive as other methods. It is vital because the technique manages to maintain its high accuracy and precision in results. Lastly, the method has been very influential and significant in the pathological world in identifying and differentiating bacteria.[9] The gram-positive and gram-negative is a very consequential process that has helped identify and select the appropriate course of action in many instances.

**Limitations of Gram Staining.** Despite the incontrovertible benefits and strengths associated with the method, it still suffers from various limitations. The gram-variable organisms produce very consequential errors and false results. Therefore, despite the pathologists being very careful in their procedures, they sometimes fall into the trap and make errors when these gram-variable organisms are involved. Also, the test is unsuitable for every bacterium, with certain bacteria showing high resistance to the gram stain.[9] For instance, the unique cell wall of acid-fast bacteria makes them a challenge in this test. Lastly, it remains highly limited in its functionality beyond the dichotomous gram-positive and gram-negative. The gram stain gives out very little information regarding bacteria beyond this and necessitates other expensive tests that may be needed to fully understand what bacteria are present [9,32-34].

**Immunohistochemistry (IHC) Staining**

This technique involves antibodies that bind to specific proteins or antigens in tissue samples, which are then visualized using chromogenic or fluorescent dyes. It is commonly used in pathology and biomedical research to identify specific markers and antigens and to understand the distribution and function of different cell types within tissues.[4] The IHC staining process involves several steps. First, the tissue sample is fixed, embedded, and sectioned into thin slices, as in H&E staining.[5] The sections are then incubated with primary antibodies binding to the target interest protein. The primary antibodies are typically detected using a secondary antibody conjugated with a fluorescent or chromogenic dye.[4,34-38] Under the microscope, the stained tissue sections show specific areas of fluorescence or color, indicating the presence and distribution of the target protein.

IHC staining is a highly specific and sensitive technique that can detect and quantify a wide range of proteins in tissue samples. It is particularly useful for identifying cell markers and antigens and characterizing the molecular changes associated with various diseases and conditions.[4] For example, IHC staining can detect the expression of specific receptors, enzymes, or growth factors in tumor samples, helping to guide treatment decisions and monitor disease progression. One limitation of IHC staining is that it requires specific antibodies that are highly selective and sensitive to the target protein. This can make it more challenging for some applications, especially when working with rare or novel antigens. Nevertheless, IHC staining remains an important and widely used technique in pathology and biomedical research, allowing researchers and clinicians to gain insights into the molecular and cellular processes underlying health and disease. Figure 5 is showing the utility of PD-L1 immunohistochemistry in cervical cancer in predicting respose to immunotherapy [4,34-38].

**Immunohistochemistry (IHC) Staining**

**Definition of IHC Staining**

The technique involves the use of a primary antibody that specifically binds to the target protein, followed by a secondary antibody that is conjugated to a detectable label, such as an enzyme or fluorescent molecule.[4] The resulting complex can then be visualized using a microscope. IHC staining is widely used in research and diagnostic settings to identify and localize specific proteins or antigens in tissue samples and characterize tissues' molecular and cellular properties.

**Staining Process**

The staining process for IHC staining typically involves the following steps: Firstly, tissue samples are first collected and fixed in a suitable fixative. Antigen retrieval involves heating the samples in a buffer solution to unmask and expose the target antigen epitopes. This step is necessary to ensure that the target antigen is accessible to the primary antibody.[4] Also, to reduce the nonspecific binding of the primary antibody, the tissue sections are treated to saturate any nonspecific binding sites on the tissue. The secondary antibody is conjugated to a detectable label, such as an enzyme or fluorescent molecule, which produces a visible signal when it binds to the primary antibody [4]. The labeled secondary antibody is detected using an appropriate substrate that produces a visible signal, such as a chromogenic substrate for enzyme-labeled antibodies or a fluorescent microscope for fluorescent-labeled antibodies. Lastly, to enhance the contrast between the tissue and the labeled cells or structures, the tissue sections may be counterstained with a dye, such as a hematoxylin, to stain the nuclei of the cells. The end result is a visual representation of the target protein or antigen in the tissue sample, which can be analyzed and interpreted by a pathologist or researcher [4,34-38].

**Applications of IHC Staining**

IHC staining has a wide range of applications in research and clinical settings, including, firstly, IHC staining can be used to identify specific biomarkers or proteins that are associated with certain types of cancer. This can aid in the diagnosis and classification of tumors and in predicting patient outcomes. Also, *IHC* staining is widely used in basic research to study the expression and localization of specific proteins in tissues and cells. It can also investigate the molecular mechanisms underlying disease pathogenesis and develop new therapeutic targets.[4] IHC staining can be used to evaluate the effectiveness of new drugs by measuring changes in the expression or localization of target proteins in response to treatment. Furthermore, IHC staining can be used to detect and localize infectious agents, such as viruses or bacteria, in tissue samples. IHC staining can be used to identify autoantibodies or immune complexes in tissue samples from patients with autoimmune diseases, such as lupus or rheumatoid arthritis. Finally, IHC staining can be used in forensic pathology to identify specific proteins or antigens in tissue samples to aid in the identification of human remains or in criminal investigations.

**Advantages and Limitations of IHC Staining**

Advantages of IHC staining include, one, IHC staining is highly specific and can target specific proteins or antigens within a tissue sample, allowing for precise identification and localization of specific cells or structures. Two, IHC staining can detect low levels of protein expression and can amplify the signal to produce a detectable signal. Three, IHC staining can be used on a wide range of tissue types and can be used to study various diseases and conditions.[4] Four, IHC staining can be used for quantitative analysis, measuring the amount of a specific protein or antigen present in a tissue sample. Five, IHC staining visually represents the target protein or antigen, allowing for easier interpretation of the results [4,34-38].

Limitations of IHC staining include one, false positives. IHC staining can produce false positive results due to the nonspecific binding of the primary or secondary antibodies. Two, false negatives since, IHC staining can produce false negative results due to low protein expression levels or poor tissue fixation.[4] Likewise, the interpretation of IHC staining results can be subjective and may require specialized training or expertise. IHC staining can be expensive, as it requires specialized equipment and reagents. Finally, IHC staining can be time-consuming, requiring multiple steps and taking several hours to complete [4,34-38].

**Conclusion**

Tissue staining techniques are important to pathologists and microbiologists, among many professions in this wide discipline. The techniques involve utilizing chemical and biological agents to selectively stain or color structures within cells and tissues for easier identification. These methods are useful for examining tissue structure, function, and pathology because they allow for the selective staining of individual structures and molecules. This data is useful in a number of ways, including research into the causes of disease, clinical practice, and patient care. A wide array of staining techniques can be applied in various instances. For instance, acid-fast staining can be used to stain mycobacterium cells and help diagnose tuberculosis. Other staining techniques, such as gram, can be critical in the classification or taxonomy of organisms by helping a reliable and quick method to group organisms. They allow pathologists the chance to analyze and visualize cells and cell structures from a microscopic level. It has led to a significant expansion in the literature that cannot be achieved in any other method. Staining in histopathology and diagnosis had undergone massive developments since the twentieth century when microbiologists discovered its various applications. It has become a very useful tool in pathology, allowing pathologists to examine the tiniest organisms and change the human course. Pathologists rely heavily on tissue staining methods because they allow for detecting pathological alterations and identifying cells and tissues. Secondly, staining techniques have played immense roles in immunohistochemistry. They have allowed for the discovery and identification of genetic markers in tissues and subsequently led to the development of disease biomarkers and potential solutions. In conclusion, diseases have created a significant revolution in pathology, diagnosis, and understanding the causative agents of various conditions.

Pathology has remained significantly unchanged despite the massive steps in almost every scientific discipline in the past twenty years in response to digitalization and technological advancements. The practice is still dominated by the microscopic evaluation of stained tissue specimens on glass slides. However, several areas offer life-changing benefits:

i. Staining has been the undoubted method of distinguishing structures in a cell. This dominance has continued despite the various shortcomings of the technique (laborious, costly, time-consuming, and destructive to specimens). However, the virtual staining of the unlabeled tissue section, which completely skips the histochemical staining procedures, offers promise.

ii. Computer-aided diagnosis has become a real possibility with the possibilities of high-output digital scanning microscopes.[14] The change is expected to change surgical pathology to include microscopic and gross photographs in the new digitized future. It is an area that promises huge changes in the industry.

iii. The advancement has seen progress beyond the silver nitrates, the traditional visual aid in pathology. The dye use is diminishing, and also the process fails in certain areas, like when dealing with argentaffin cells.[15] The Grocott-Gomori and Gomori Reticulin are some approaches being mooed as possible solutions. These developments will revolutionize the industry.

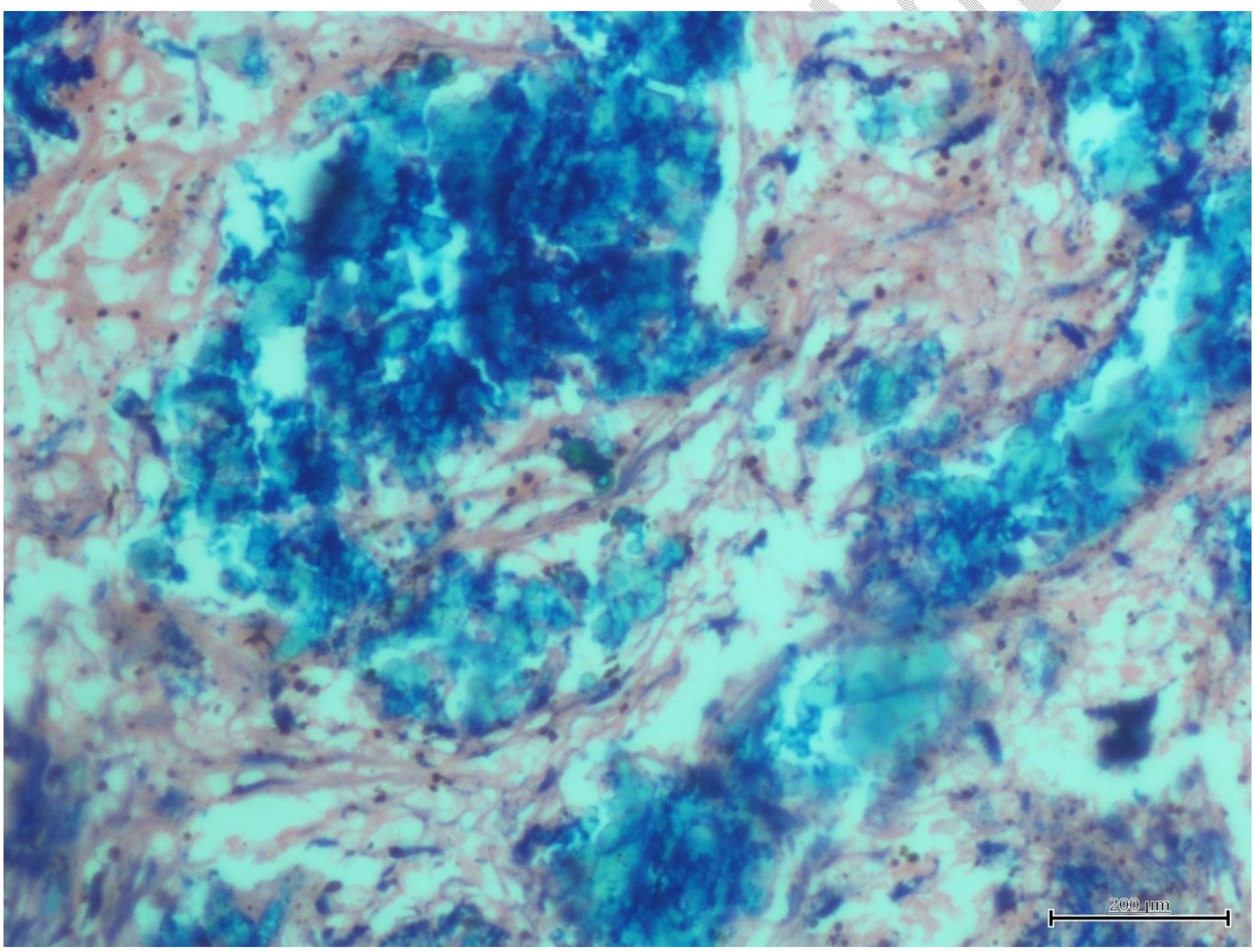


Fig. 1: Perls Prussian Blue staining in a splenectomy specimen to demonstrate gamna gandy bodies which can be seen sickle cell anemia. The staining technique is used to demonstrate the presence of iron in tissue or cell samples.

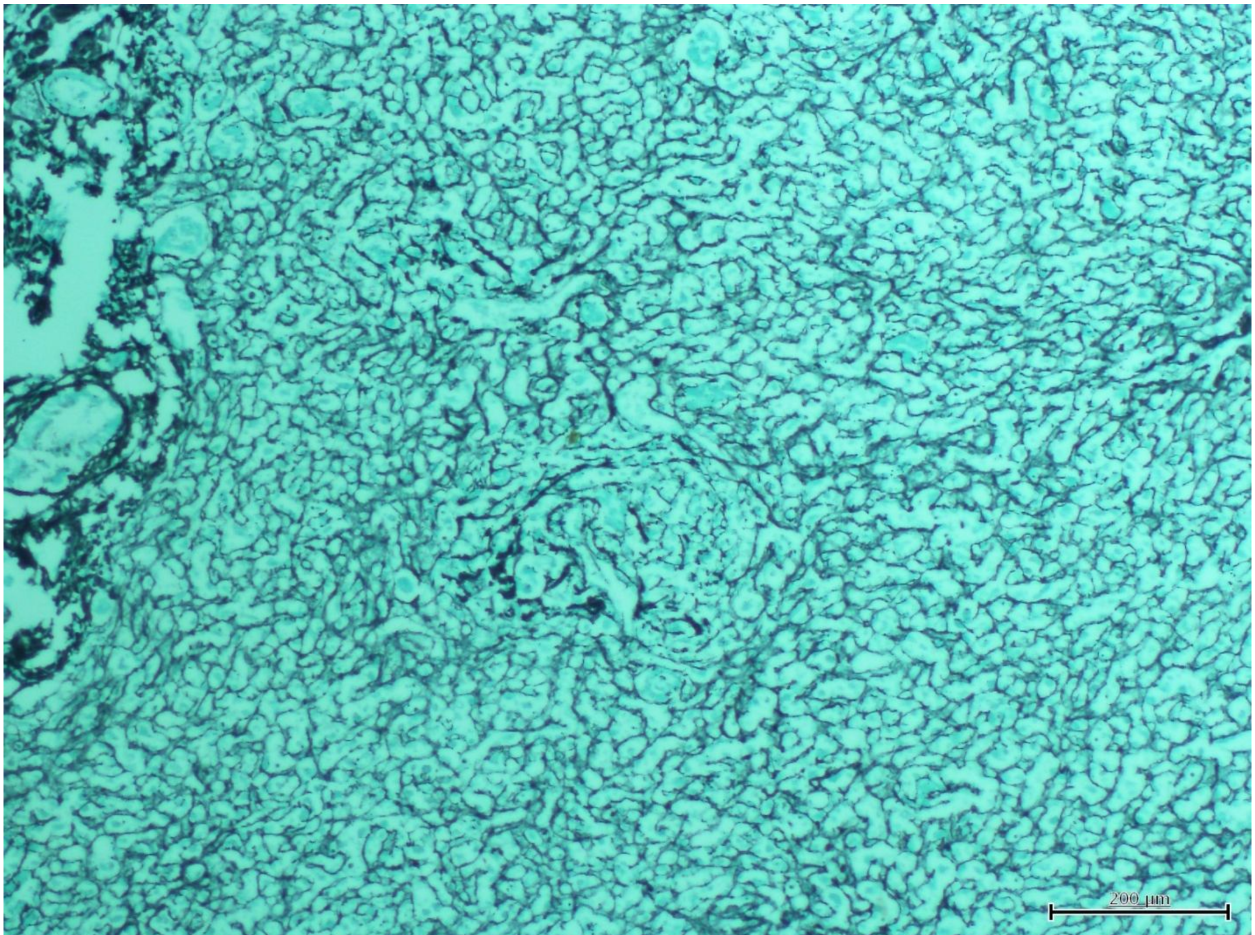


Fig. 2: Reticulin stain used in assessing liver architecture. This staining technique highlights type III collagen and helps to demonstrate the thickness hepatocyte plates. Hepatocellular carcinoma usually show loss of reticulin fibres thus helping in differentiating benign proliferations from malignant hepatocellular carcinoma.

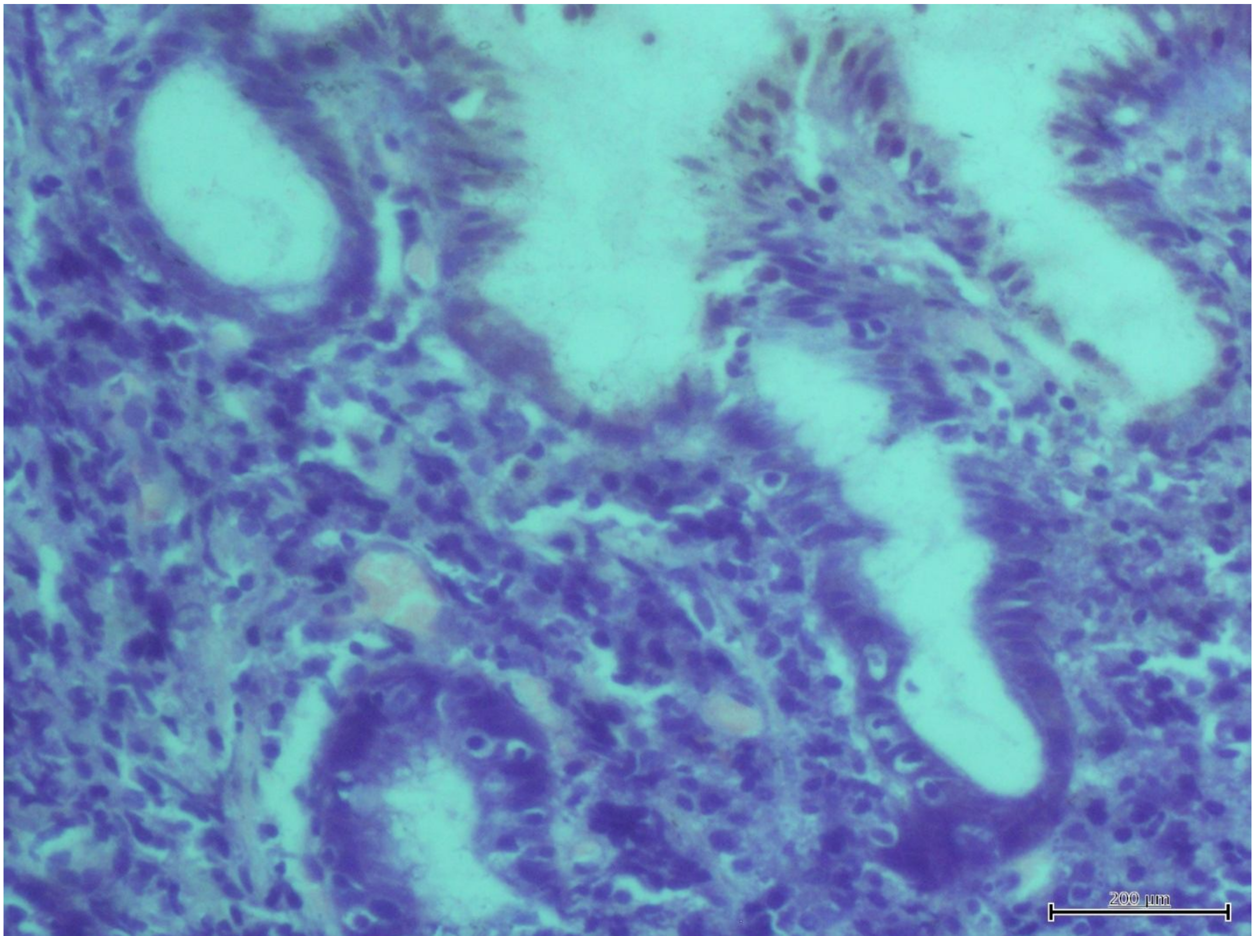


Fig. 3: Giemsa staining in a gastric biopsy used to demonstrate H. pylori organisms in the gastric crypts.

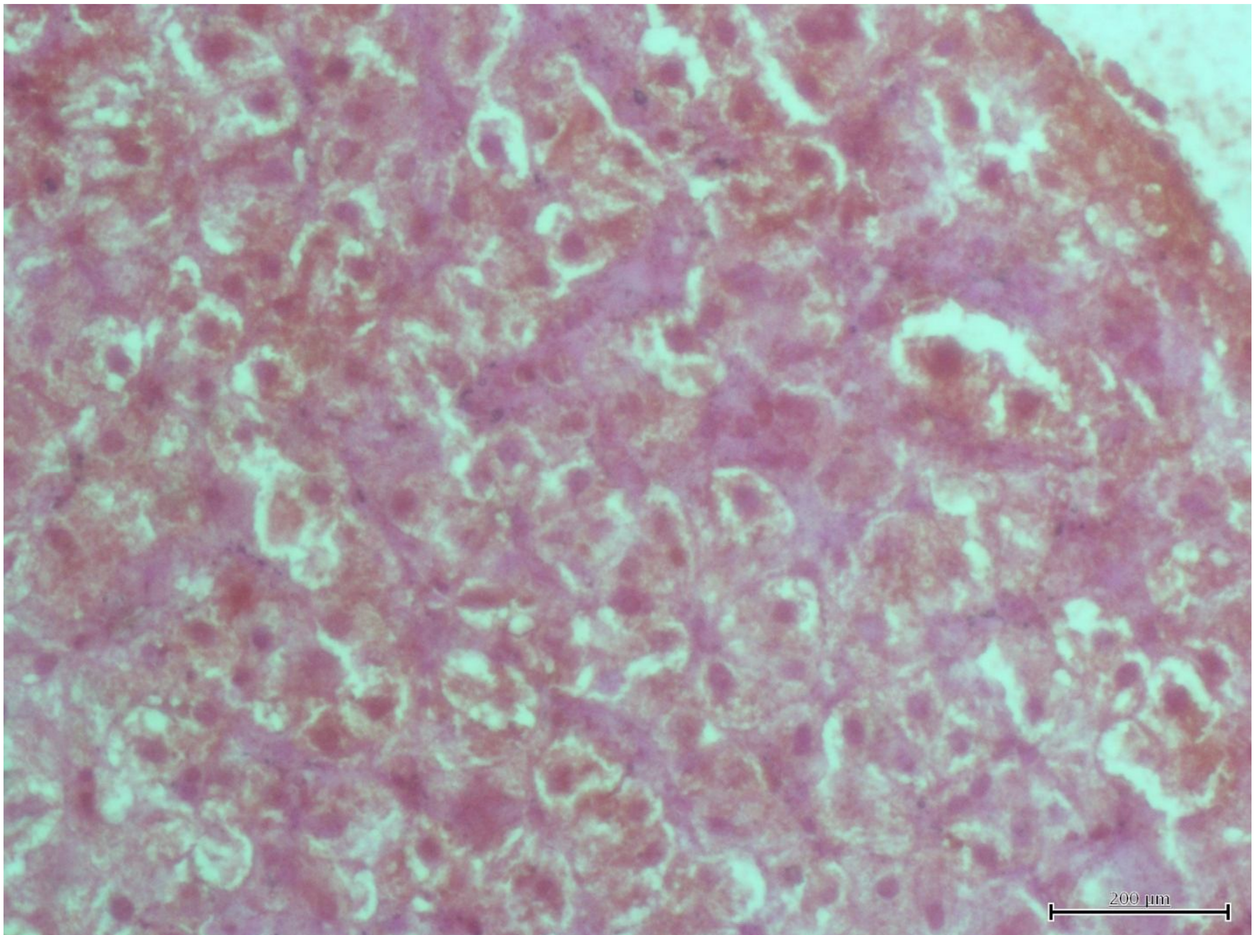


Fig. 4: Shikata Orcein staing technique used in demonstrating HBsAg viral inclusion bodies in hepatocytes.

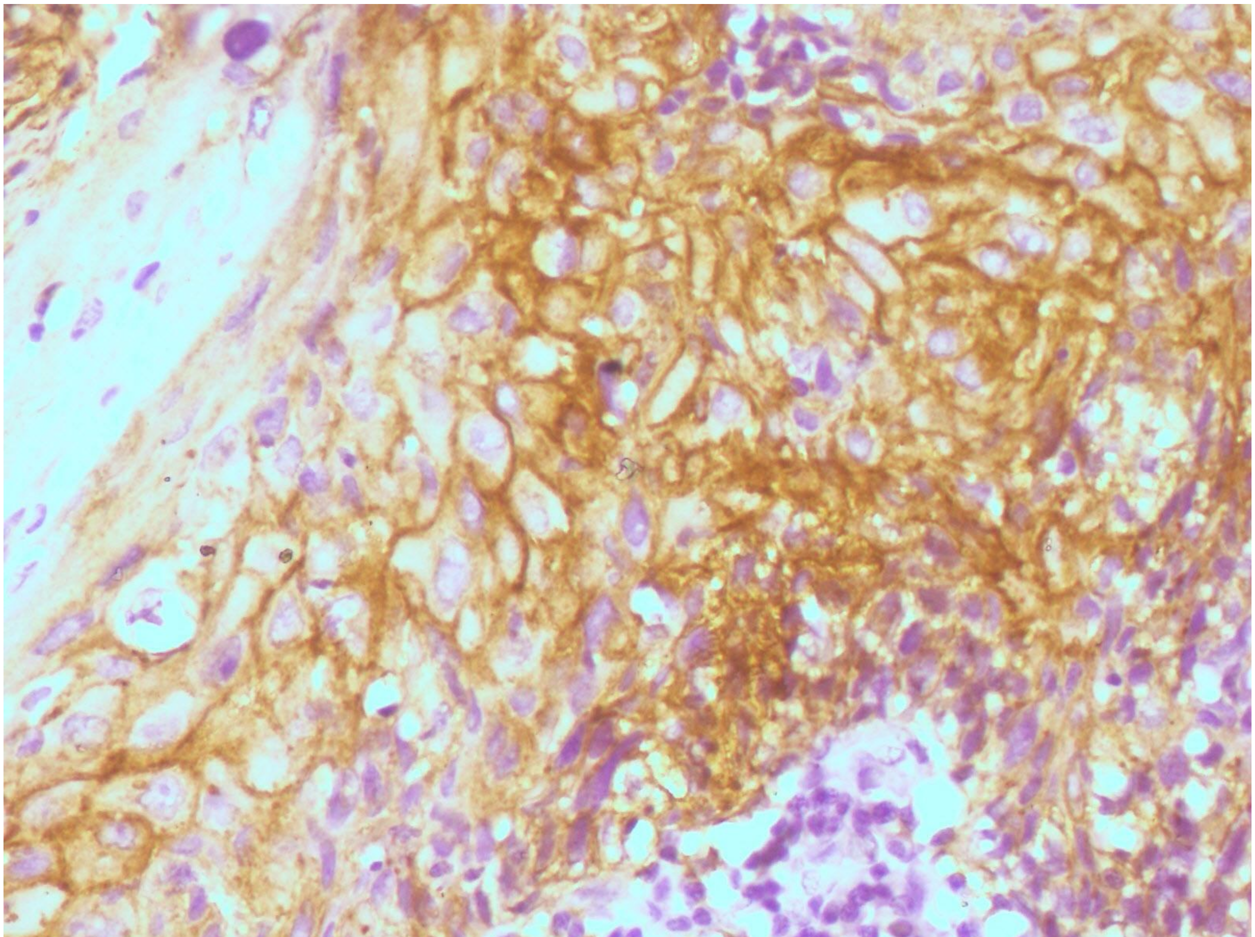

Fig 5. PD-L1 immunohistochemical staining showing a strong staining intensity and this predicts response to immunotherapy with pembrolizumab.

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