The Bitesize Guide to Special Stains for Histology

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This eBook was created using Bitesize Bio blog articles authored by Dr Nicola Parry and was edited by Dr Amanda Welch and Dr Martin Wilson

Introduction

This eBook provides the bioscientist with an overview of the alternative staining techniques and procedures known as the “special stains.” Eleven of the most common special stains are described in this book. It covers their history and discovery, general principles of each stain, and their uses in the research and diagnostic labs.

The most commonly used stain in histology labs is haematoxylin and eosin (or H&E) representing the “bread and butter” stain for most pathologists who diagnose disease, and for researchers who work with many tissue types. It highlights the detail in tissues and cells, using a haematoxylin dye to stain cell nuclei blue, and an eosin dye to stain other structures pink or red.

Although H&E is an essential everyday stain for many pathologists and researchers, sometimes a little extra help is required to reach a diagnosis, or further evaluate a tissue- usually to differentiate components that have already been seen in H&E-stained tissue sections but need to be definitively identified.

Special Stains

This is where the so-called “special stains” come in handy. This term describes a large number of alternate staining techniques and histochemical procedures that are used in situations where H&E cannot provide all the information needed by a pathologist or researcher.

It’s unclear exactly how the term “special stains” first arose in the world of histology, but it refers to empirical and histochemical staining techniques that significantly contributed to the advancement of histology in the late 19th century.

In a nutshell, these stains are “special” because they are not routine, simple as that. Therefore, special stains refer to any staining technique other than H&E. So, when we talk about special stains, we are referring to stains applied for a single purpose.

These techniques use a variety of staining methods to more readily visualize components of a tissue using light microscopy. In principle, they work by taking advantage of intra- and extracellular chemical reactions between the tissue components and dyes. Typically, they use a chemical or dye with an affinity for whatever is under investigation, enabling specific tissues, structures, or even microorganisms to be stained.

Why Do We Use Special Stains?

Routine H&E-staining remains the cornerstone of histological tissue examination. This stain is important for providing detail of tissue and cell structure, as well as differentiation of cellular constituents, especially the cytoplasm and nucleus.

However, examination of H&E-stained tissues may raise questions, such as;

- What type of tissue is present, and what is its distribution?
- Which tissue component is that?
- Is that an organism?!

This is where special stains come in. They represent a variety of techniques and dyes with a more limited staining range, so they can be used to highlight certain features. This allows target substances to be identified based on their;

- Chemical character: For example – calcium, iron, copper, melanin, lipids, glycosaminoglycans, hemosiderin, DNA and RNA.
- Biological character: For example – connective tissues, nerve fibers and myelin.
- Pathological character: For example – bacteria, fungi and amyloid.

In this way, special stains can be used diagnostically, as well as in biomedical research, either to detect the presence of specific tissue structures or substances or to allow a more detailed morphological evaluation of these components and their distribution within a specimen.

Typically, H&E-stained sections of the specimen have been evaluated prior to the decision to apply a special stain. In diagnostics, for instance, if a pathologist sees changes in a tissue to suggest a diagnosis of tuberculosis, an acid-fast stain can be used to confirm this.

Similarly, in a research setting, an investigator evaluate liver specimens for evidence of cancer development and may, incidentally, discover variable deposits of extracellular pink material. In this case, a Congo red stain would be a helpful follow-up stain to determine if the material was amyloid. Conversely, the researcher may start out wanting to investigate whether amyloid deposition was occurring. In this case, Congo red staining would likely be requested simultaneously with H&E, because both stains would be needed to fully investigate the original scientific hypothesis.

The Need for Positive Controls

Generally, when special staining is performed on a section of tissue, a positive control section is also stained. This involves simultaneous staining of a section of tissue known to contain the substance in question. This will be given to the pathologist or researcher at the same time as the test tissue section and serves various purposes. In particular, the positive control helps to determine whether the special stain technique is working and also serves as a reference of what the substance should look like in the test tissue section.

Empirical or Histochemical?

Some special stains, such as the trichrome stain for connective tissue, are specifically empirical. That is, they are used only to produce differential coloration of

various cell and tissue components. Because of different mordants and differentiations used in these stains, it is difficult to reproduce specific color densities. This makes it difficult to standardize them (a “mordant” is an ion, usually metal or halide, that binds the stain or dye to the target organism or component).

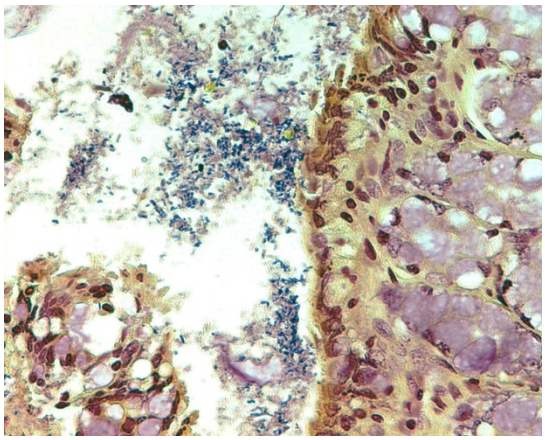
On the other hand, histochemical stains, such as Periodic acid-Schiff stain for mucopolysaccharides, are very specific for a single molecular species. Therefore, these stains can be standardized and repeated with accuracy.

Although immunohistochemistry and *in situ* hybridization are technically forms of special stains, they represent a class of techniques based on specific chemical reactions. Their specificity is such that they can recognize very precise targets within a tissue, namely proteins or DNA/RNA sequences.

Many histologists prefer to distinguish these techniques from the more usual special stains, and often categorize them as “Advanced Stains.”

Let us now look at some of the special stains in more detail, starting with those used to identify bacteria, a stain for mast cells, and then a number of stains for tissue and cellular components.

Gram Staining for Bacteria



The Gram stain is one of the most common special stains used in the lab. It's a differential staining technique that represents an important initial step in the characterization and classification of bacteria using a light microscope. It is named after a Danish scientist, Hans Christian Gram, who developed the method in 1884 to distinguish between two types of bacteria that caused similar symptoms.

The Difficulty with Bacteria

Bacteria are the most difficult organisms to detect in H&E-stained sections of tissue. However, when certain histologic changes are suggestive of a bacterial infection (such as a consistent pattern of inflammation) a Gram-stained section of the tissue can then be helpful to determine if bacteria are indeed present.

The stain classifies organisms as either:

- Gram-positive (e.g., *Staphylococcus* spp., *Streptococcus* spp.)
- Gram-negative (e.g., *Escherichia coli*, *Salmonella* spp.)

The Bacterial Cell Wall

Although Gram-positive and Gram-negative bacteria have similar internal structures, they differ with respect to the composition of their cell wall. Gram-negative bacteria ten to have a more structurally and chemically complex cell wall.

A couple of major differences in the cell wall:

- Peptidoglycan: A mesh-like layer of sugars and amino acids
    - Present in both Gram-positive and Gram-negative bacteria
    - Thicker in Gram-positive bacteria
- Lipopolysaccharide: A lipid-polysaccharide layer external to the peptidoglycan
    - Present only in Gram-negative bacteria

General Principles of the Gram Stain

The procedure involves four basic steps:

1. Initial staining with crystal violet. This is a basic dye that similarly stains both Gram-positive and Gram-negative organisms.
2. Treatment with an iodine and potassium iodide solution (mordant) that serves to fix the crystal violet stain. Both Gram-positive and Gram-negative organisms remain purple after this step.
3. Decolorization with a mixture of alcohol and acetone. This is the differential step: Gram-negative bacteria become destained, while Gram-positive bacteria retain the color of the crystal violet-iodine complex.
4. Counterstaining with fuchsin (red) or safranin (pink). Because the Gram-positive bacteria are already stained purple, they are not affected by the counterstain. However, the now colorless Gram-negative bacteria do become stained.

Influence of the Bacterial Cell Wall

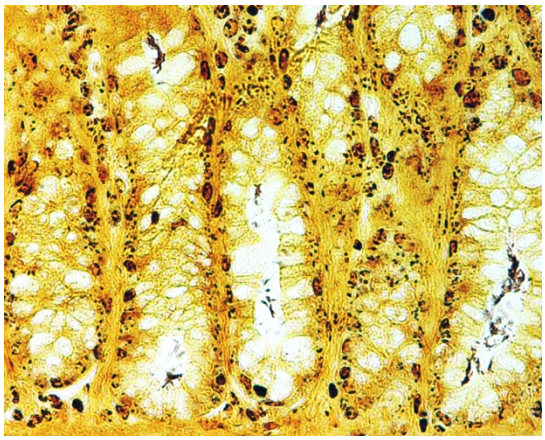
The difference in staining between Gram-positive and Gram-negative bacteria is based on their ability to retain the color of the violet stain used during the reaction.

The lipopolysaccharide layer of Gram-negative bacteria holds the key to this. It is disrupted in the decolorization step. This allows the original crystal violet stain to leach out, and the basic fuchsin or safranin counterstain to be taken up.

The thicker peptidoglycan layer of Gram-positive bacteria, on the other hand, allows the crystal violet stain to be retained.

Bacteria	**Color in Gram-stained sections**
Gram-positive	Blue-black
Gram-negative	Red-pink

Warthin-Starry Stain for Microorganisms



Some Gram-negative organisms do not stain well using the Gram stain technique. These include spirochaetes (such as *Helicobacter, Leptospira, Borrelia,* and *Treponema* spp.), as well as small bacilli (including *Campylobacter, Bartonella,* and *Legionella* spp.).

The Warthin-Starry stain is considered the best staining technique to detect these types of organisms. It was first developed in 1920 by American pathologists Aldred Scott Warthin and Allen Chronister Starry, and is commonly referred to as a 'silver stain' because it is a silver nitrate-based staining method.

General Principles of the Warthin-Starry Stain

The procedure involves two basic steps:

1. Slides of tissue sections are immersed in an acidified aqueous solution of silver nitrate (optimum pH 3.5 to 4.0). Two reactions occur during this time. Firstly, large numbers of silver ions form bonds with protein molecules throughout the tissue. Secondly, smaller numbers of silver ions are reduced to silver atoms at specific sites in the tissue- within or on the surfaces of organisms. These metallic silver deposits are tiny particles known as "nuclei" (but these are not the same as cell nuclei).

2. Slides are immersed in a reducing solution that typically contains hydroquinone, gelatin, and a lower concentration of silver nitrate. This solution acts as a type of "developer": the gelatin sequesters silver ions, slowing the rate at which they are reduced to metal by the hydroquinone. This slow reduction of the silver ions is catalyzed by the nuclei that were formed in the first step. Eventually, these tiny nuclei enlarge as more and more silver deposits on the organisms, until enough is present to make the organisms visible as black objects under the microscope.

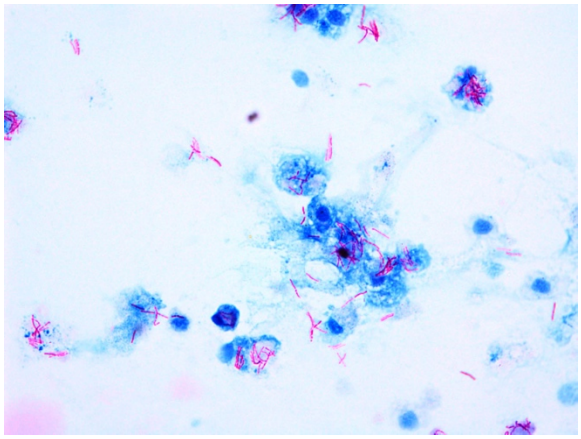
Consequently, the end product of the reaction is such that the organisms stain dark brown to black. The lower concentrations of silver deposits stain golden brown and provide a contrasting background staining of the architecture of the tissue.

Who Uses Warthin-Starry Stain?

This stain is used widely for both diagnostic and research purposes. For example, researchers investigating diseases, such as leptospirosis, cat scratch disease, and Helicobacter-associated gastric cancer, may routinely examine WS-stained tissue sections to detect the presence of organisms in tissues of interest.

In diagnostic labs, pathologists often use this stain in conjunction with a Gram stain to help answer questions that may arise after findings in routine H&E-stained sections suggest bacterial involvement.

Acid-Fast Stain for Microorganisms



Another special stain used to identify microorganisms is the Acid-Fast (AF) stain. This differential stain identifies acid-fast bacterial organisms, such as members of the genus *Mycobacterium* and *Nocardia.*

The Discovery of Tuberculosis

German scientist and physician, Robert Koch, was a Nobel Laureate in Medicine and a founder of the science of bacteriology. In 1882, his discovery of *Mycobacterium tuberculosis* (the bacterial agent responsible for tuberculosis) was a major event in the history of medicine. At this time, he described the appearance of the bacterium as a result of a complex staining procedure that became the original AF stain.

The Ziehl-Neelson Method

Although there are various types of AF staining techniques, the Ziehl-Neelson (ZN) method is used most widely. This technique was first described in the 1800s by two German doctors: bacteriologist Frank Ziehl and pathologist Friedrich Neelson. Both modified Koch's staining technique to further improve on it. Ziehl was the first who used carbolic acid (phenol) as the mordant. Neelsen kept Ziehl's mordant, but changed the primary stain to the basic fuchsin. In the mid-1890s, this technique was named the ZN method.

General Principles of the Stain

Acid-fast bacteria have a cell wall consisting of mycolic acid, fatty acids, waxes, and complex lipids. This produces a waxy cell wall that is almost impermeable, and therefore resistant to most compounds, including the routine bacterial special stains, such as the Gram stain. A special staining technique is therefore needed to identify these organisms.

This is where the ZN method comes in.

Primary Stain

Carbol fuchsin is the primary stain in this technique. This is lipid-soluble and contains phenol to help drive the primary stain into the waxy cell walls of these bacteria. Heat may also be used ("hot staining") at this stage to soften the cell wall and further help the stain permeate.

Decolorizer

The slide is now rinsed with a strong acid-alcohol decolorizer. This strips the stain from all non-acid-fast cells. However, the acid cannot penetrate the cell wall of acid-fast bacteria (hence the term "acid-fast").

Counterstain

After decolorization, the slide is counterstained with methylene blue. The decolorized non-acid-fast cells are able to take up the methylene blue counterstain, and therefore stain blue. The rod-shaped acid-fast bacteria that resisted decolorization, however, will stain red-pink, the color of the initial carbol fuchsin stain.

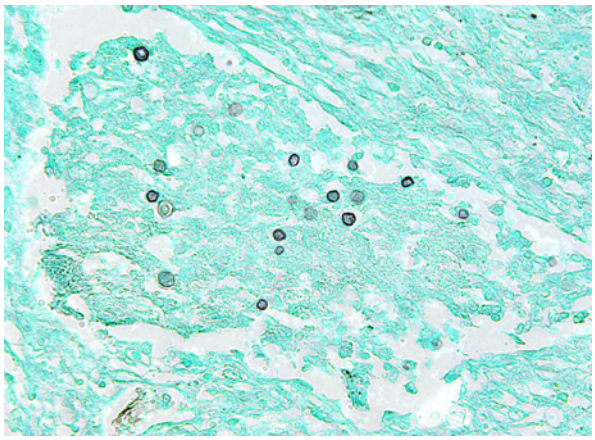
Who Uses AF Staining Methods?

The ZN method continues to remain a reliable and effective way to demonstrate the acid-fast bacteria, and it is used for both research and diagnostic purposes.

Researchers studying acid-fast organisms, such as members of the genus *Mycobacterium and Nocardia,* will use this staining technique routinely to examine sections to evaluate the bacteria in certain tissues.

The AF stain is also used diagnostically by pathologists to identify these organisms and is an especially important technique in the diagnosis of tuberculosis. It is also used in the diagnosis of other mycobacterial diseases, including leprosy due to infection with *Mycobacterium leprae.* Other mycobacterial organisms, the so-called "atypical species," can also cause tuberculosis-like infections, especially in immunosuppressed patients. *Mycobacterium avium-intracellulare* complex, for example, often causes systemic infections in people with HIV/AIDS.

Gomori's Methenamine Silver (GMS) Stain for Microorganisms and Fungi



The final special stain for microorganisms covered in this eBook is Gomori's Methenamine Silver (GMS) stain.

What Does GMS Stain?

1. Fungi

GMS is probably best known for staining fungal organisms. Fungi are generally relatively large and morphologically diverse and can occur in tissues in various forms: hyphae, endosporulating spores, budding yeasts, or a combination of these forms.

Classic Features

Some fungal infections are caused by a species of fungus with very characteristic morphology and can therefore be diagnosed relatively easily on H&E-stained sections when their classic features are seen. These include species of *Cryptococcus,* as well as others such as *Histoplasma, Blastomyces, Coccidiomyces, Rhinosporidium,* and *Prototheca*.

When we Can't Rely on Histology Alone

Other infections, however, may be due to one of many species of a genus. Because these all appear similar in tissue sections, histology alone cannot definitively identify the species involved, but can at least help to broadly categorize the fungal disease. This is the case with aspergillosis and candidiasis, for example.

Additionally, some infections may even be caused by a number of fungi belonging to different genera. These different fungi may appear similar within tissue, so the exact organism cannot be definitively identified. Examination of H&E-stained sections can at least help to broadly categorize the fungal disease such as phaeohyphomycosis or zygomycosis.

Difficulties with Fungal Identification

Microscopic evaluation of tissues is a quick and easy way to identify fungal organisms. These various forms can often be identified in H&E-stained tissue sections. However, although H&E is the stain of choice to identify naturally pigmented fungi (and the nuclei of yeast-like cells), it is not advisable to use this stain in isolation to diagnose fungal infections. This is because some fungi may be easily overlooked in H&E-stained sections, especially if not naturally pigmented, or if they are present in low numbers. It can also be difficult to differentiate poorly stained fungi from tissue components in H&E-stained sections. Additionally, fungal identification may be difficult if classic features are either absent or altered—the latter of which may occur in association with fungal therapy.

So, when histological evidence of granulomatous inflammation or actual granulomas is found in tissues, special stains are generally performed to check for the presence of infectious organisms that can cause such changes- namely fungi and acid-fast bacteria.

2. Other Organisms

GMS can be a useful stain to identify other organisms, too.

Algae	Such as *Prototheca* spp. and *Chlorella* spp.
***Pneumocystis* spp.**	Stains their cyst wall
Microsporidian organisms	Stains their spore coat
Some bacterial organisms	*Nocardia* spp., *Mycobacterium* spp., and non-filamentous bacteria with polysaccharide capsules, such as *Klebsiella pneumoniae* and *Streptococcus pneumoniae.*

General Principles of the Stain

GMS is a chromic acid, sodium bisulfite stain.

The initial step of the GMS stain is similar to that of the Periodic acid-Schiff stain (PAS, covered later in the book) in that it uses chromic acid as an oxidizing agent. During this step, chromic acid oxidation forms aldehydes from fungal cell wall mucopolysaccharide components.

The tissue is then treated with methenamine silver nitrate solution. During this step, the aldehyde groups react with the silver nitrate, reducing it to metallic silver, which appears black.

In contrast to PAS stain, however, chromic acid is a stronger oxidizing agent than periodic acid and will oxidize some of the aldehyde groups further. This produces a substance that cannot react with silver ions. The collagenous background in tissues, therefore, does not stain.

When a counterstain of light green solution is used, the fungal elements stain black with sharp margins and a cleared center against a light green tissue background.

GMS Versus PAS

GMS and PAS are the two most common stains used to check for fungi in tissues. Both are considered “broad spectrum” fungal stains, because most fungi can be readily seen with either stain.

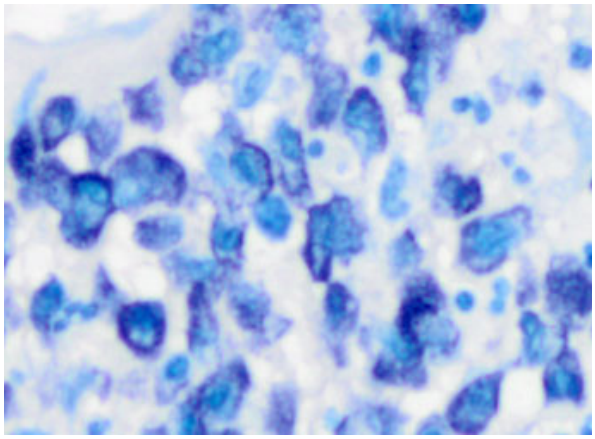
One advantage of GMS is that it produces better staining contrast in tissues sections and detects even degenerated and dead fungi that may not be detected by PAS stain. This makes GMS a better choice for general screening of tissues for fungal infection.

Who Uses GMS Stain?

This stain is used widely for both diagnostic and research purposes. For example, researchers studying fungal organisms may routinely examine GMS-stained sections to evaluate certain fungal elements in tissues of interest.

In diagnostic labs, GMS tissue staining is often used in combination with microbiologic culture for diagnosis of fungal infections in people and animals, because the presence of fungal organisms in tissues indicates an invasive infection. Therefore, pathologists will often use this stain to help answer questions after examining routine H&E-stained sections containing changes suggestive of fungal infection.

Toluidine Blue Stain for Mast Cells, Cancer Screening and Forensics



Toluidine blue (TB) is a polychromatic dye that can absorb different colors depending on how it binds chemically with various tissue components.

It first emerged in 1856, courtesy of a British chemist called William Henry Perkin. Although he was working on the synthesis of quinine, Perkin instead produced a blue substance with good dyeing properties. Initially, it became known as aniline purple and was mostly used in the dye industry. Later, it became known as TB, and began being used for medical purposes, in particular as a histological special stain to highlight certain components.

What Does it Stain?

This basic dye selectively stains acidic tissue components. It has a specific affinity for nucleic acids, and therefore binds to nuclear material of tissues with a high DNA and RNA content.

General Principles of the Stain

TB stains tissues via the phenomenon of metachromasia. Many dyes can show metachromasia, but the thiazine group dyes (such as TB) are especially good for this type of staining. This phenomenon refers to how a dye stains tissue components a different color from the dye solution and the rest of the tissue. In the case of TB, this strong, acidophilic, blue dye stains nuclei dark blue, but will stain mast cell granules and polysaccharides violet.

One advantage of using TB is that it is a relatively simple stain to perform, and the staining process is much quicker than that of routine H&E-staining.

Who Uses Toluidine Blue Stain?

This stain is used widely for both diagnostic and research purposes. In diagnostic labs, TB is widely used by pathologists to highlight mast cell granules. This stain is particularly helpful when they need to evaluate patients with pathological conditions that involve mast cells, including cancers, allergic inflammatory diseases, and gastrointestinal diseases such as irritable bowel syndrome.

It is also used to highlight tissue components, such as cartilage or certain types of mucin. Additionally, it can be used as part of the screening process for certain cancers, such as oral cancer—because it binds the DNA of dividing cells, precancerous and cancerous cells take up more of the dye than normal cells.

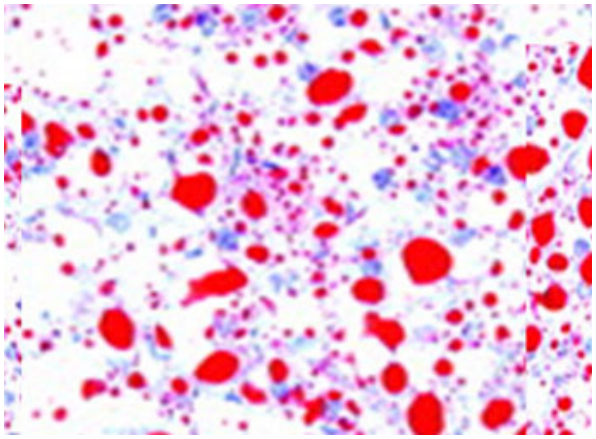
From Research Labs to Forensics

Similarly, researchers investigating any of these diseases or tissue components may also routinely examine TB-stained tissue sections. Other researchers may also use the stain simply because they come across something in tissue sections that needs to be identified.

It may also be useful in electron microscopy for staining thin sections of resin-embedded tissues to help with the orientation and visualization of samples. Although TB stains everything in the sample, structural details are especially prominent because the sections are so thin.

TB is also used in sexual assault forensic medical examinations. Because the dye is preferentially taken-up by injured cells, it can be used to identify damaged tissue in the genital tract.

Oil Red O Stain for Fats and Lipids



Oil Red O ('ORO') is used to demonstrate the presence of fat or lipids in fresh, frozen tissue sections. Introduced by French in 1926, ORO is a fat-soluble diazo dye, and is classified as one of the Sudan dyes that have been in use since the late 1800s.

Like most stains used to detect lipids, ORO isn't a true special stain, because it can't form bonds with lipid components. It is actually a pigment that functions as an oil-soluble colorant, and the technique represents a physical method of staining.

Why Do We Need Oil Red O?

Lipids in tissues pose a bit of a challenge to histologists and pathologists for a couple of reasons:

1. Chemically, they are relatively unreactive—even unsaturated lipids have few binding sites for stain molecules.

2. To add insult to injury, lipids don't stay in tissues after routine processing. Xylene and alcohol solvents tend to dissolve and leach them out during the paraffin embedding process. So, by the time H&E stain is applied, the presence of lipids can only be suspected by their absence—that is, the unstained 'holes' that are left behind.

   This isn't a problem in plastic-embedded tissues that are processed without lengthy solvent immersions. However, most labs still use paraffin wax embedding because it is faster, cheaper and well-suited to automatic tissue processors.

General Principles of the Stain

The basis for staining lipids with an oil-soluble dye lies in its increased solubility in fatty substances as opposed to the dye solvents that are used in routine tissue processing.

The choice of solvent for this reaction is also critical, since it must be able to extract excess dye without dissolving the lipid to be stained- propylene glycol is the preferred solvent for this technique.

The end result is that fat and lipids in tissue sections stain bright red, and nuclei stain blue. Although other stains are available to help detect the presence of lipids in tissues, the intensity of its red coloration makes ORO the preferred choice.

Who Uses Oil Red O Stain?

This stain is used widely for diagnostic, research, and forensic purposes.

In Diagnostic Labs

Pathologists use ORO to help diagnose various conditions in which fat may appear in abnormal locations. For instance, bone fractures or crush injuries to fatty regions of the body may release fat into the bloodstream, leading to fat emboli that can be fatal- these emboli are detected using ORO. It is also useful to identify tumors, such as lipomas and liposarcomas that arise from fat cells. Deposits of fat may also appear in the liver and kidney in a variety of pathological conditions.

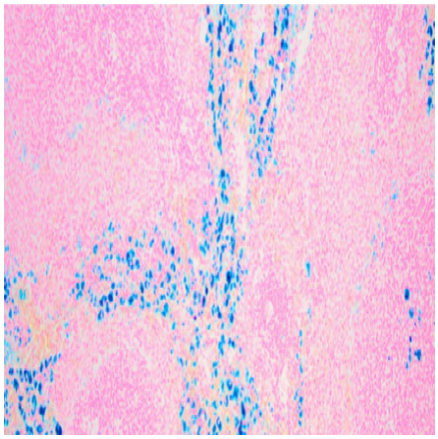
In Research Labs

Researchers investigating any of the above medical conditions, as well as those involved in research in fat metabolism, may also routinely examine ORO-stained tissue sections to detect the presence of fat in tissues of interest. Others may simply use the stain if they come across areas in H&E-stained tissue sections leading them to suspect the presence of fat.

In Forensic Labs

ORO is also used in forensic pathology as a reagent for fingerprint development. Detecting fingerprints on porous surfaces that have been exposed to moisture is difficult as the amino acids from the prints will dissolve in water. In 2004, Alex Beaudoin discovered a technique using ORO as a reagent to enhance latent prints produced by the lipids found in fingerprints. This makes it particularly easy to reveal latents on wet, porous surfaces such as paper and cardboard.

Prussian Blue Stain for Iron in Tissue and Cells



Prussian blue (PB) was actually the first synthetic color to be discovered during the Industrial Revolution. It was developed accidentally in 1704 by a chemist who was trying to produce another color.

It wasn't used as a histochemical stain until 1867 when its original formula was described by German pathologist, Max Perls - hence why is often known as 'Perls' Prussian blue'.

The PB stain is not a true special staining technique but rather, a histochemical reaction.

What Does it Stain?

PB is used to detect the presence of iron in tissues. It is an extremely sensitive test, and can even detect single granules of iron in cells.

Although iron is essential for life, it is also toxic due to its ability to form free radicals that can damage cells. So the body must protect itself from this element, and it does so by using iron-storage proteins. Haemosiderin is one type of iron-storage complex that is found inside cells. It is mostly found within phagocytic macrophages, and is especially prominent in these cells following haemorrhage, when haemoglobin breaks down.

Microscopically, the presence of iron is therefore identified by demonstrating iron-containing haemosiderin that shows up as a granular brown pigment in cells when examined with H&E stain. Although visible with H&E, other pigments can stain a similar color, so haemosiderin needs to be differentiated from other brown pigments using the PB histochemical staining technique.

General Principles of the Stain

Various staining protocols have emerged since Perls' original description, but all serve to achieve the same result, namely the demonstration of iron in tissues.

The procedure involves three basic steps:

1. Tissue sections are treated with hydrochloric acid to denature the binding proteins of the haemosiderin molecule, and thereby release ferric (3+) ions.

2. Potassium ferrocyanide is then introduced. The ferric ions combine with this solution, resulting in the formation of ferric ferrocyanide, an insoluble bright blue pigment (otherwise known as Prussian blue).

   Although the hydrochloric acid and potassium ferrocyanide can be introduced as separate solutions, most formulations now typically use them in combination.

3. If required, the PB reaction can then be followed by a red counterstain, such as neutral red or safranin O.

Consequently, the end product of the reaction is such that iron in tissue sections is seen as blue or purple deposits, while other tissue components, such as nuclei and cytoplasm, are highlighted red by the counterstain.

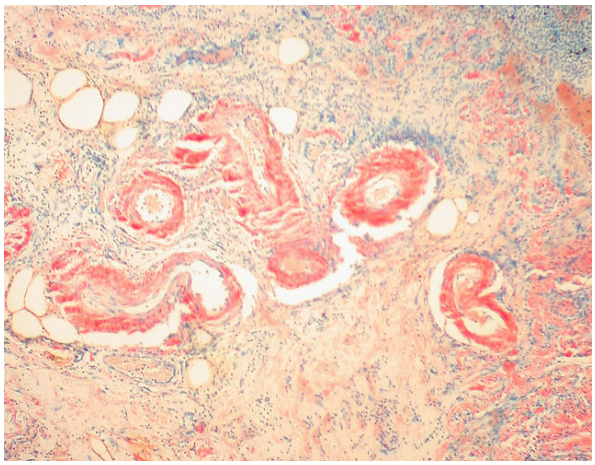
Who Uses Prussian Blue Stain?

This stain is used widely for both diagnostic and research purposes. In diagnostic labs, PB is widely used by pathologists to detect the presence of iron in biopsy specimens, especially in tissues such as bone marrow and spleen.

This procedure is particularly helpful when they need to evaluate patients with pathological conditions that involve haemosiderin deposits. In addition to haemorrhage, this can occur in patients with haemolytic anaemia, as well in conditions such as haemochromatosis (where excessive amounts of iron may form in organs due to iron overload), some liver diseases, and in the lungs of patients with congestive heart failure.

Similarly, researchers investigating any of these types of diseases may also routinely examine PB-stained tissue sections to detect the presence of iron in tissues of interest. Finally, others may use the stain simply because they come across a brown granular pigment in tissue sections that needs to be identified.

Congo Red Stain for Amyloid and Alzheimer's Disease



Did you know that Congo red (CR) originally started out as a textile dye? It belongs to the azo class of dyes- synthetic compounds that are among the most popular dyes used in the clothing and fashion industry. CR is a benzidine derivative that can react with structural polysaccharides including the cellulose in textiles.

What Does it Stain?

In the histology world CR is used to stain amyloid. This is an abnormally folded, fibrillar protein that deposits in extracellular spaces in organs under certain pathological conditions. As it accumulates, it progressively replaces the normal tissue elements, and eventually results in loss of function of vital organs, and ultimately, death.

What's in a Name?

The exact origin of the name of this stain is unclear, but evidence certainly hints at a connection with the West Africa Conference that was held in Berlin around the time of the discovery of this dye. This diplomatic meeting was a result of a trade dispute between European powers regarding the Congo River Basin in Africa.

The Only Histology Stain Named by a German Dye Company

So it's thought that a Berlin dye company gave the dye its name because the Congo was in the forefront of everyone's minds at that time! Although CR was used to stain tissue sections after it was first discovered, it wasn't until a few decades later, in 1922, that a German physician, Hermann Bennhold, found that it also binds to amyloid. He realized that an intravenous injection of CR in patients with amyloid led to loss of the dye from plasma, and its accumulation in amyloid deposits.

'Apple Green' Amyloid Plaques

A few years later, in 1927, it was a Belgian neuropathologist, Paul Divry, who was the first to describe the 'apple green' birefringence of amyloid plaques of patients with Alzheimer's disease when stained with CR and examined under polarized light.

General Principles of the Stain

Amyloid is similar in structure to cellulose, therefore it behaves similarly in its chemical reactions. It is a linear molecule, which allows azo and amine groups of the dye to form hydrogen bonds with similar hydroxyl radicals of the amyloid.

In H&E-stained tissue sections, amyloid appears as an amorphous, glassy, eosinophilic material. Since this can be confused with some other materials, CR staining is needed to identify it.

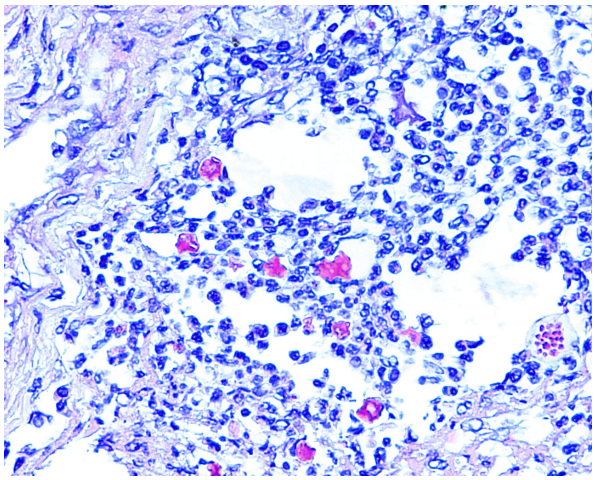
When examined using regular bright-field microscopy, CR-stained amyloid appears pale orange-red. However, the bright field appearance alone is not diagnostic for amyloid, because small deposits may be difficult to see. CR-stained tissue sections must therefore be examined under polarized light allowing the characteristic ‘apple green’ birefringence to be seen which is diagnostic for the presence of amyloid.

Who Uses CR Stain?

The CR staining technique is a reliable and effective way to demonstrate amyloid deposits in tissues, and it is used for both research and diagnostic purposes.

Researchers studying amyloidosis (disorders of diverse origin in which deposits of amyloid proteins are found) will routinely use this staining technique to detect the presence of amyloid in their tissues of interest.

Periodic Acid-Schiff Stain for Polysaccharides and Basement Membranes



Even after all these decades, CR still remains the gold standard test used by diagnostic pathologists to identify amyloid in tissues of patients with these conditions, perhaps the most widely known of which is Alzheimer's Disease. Periodic acid-Schiff (PAS) is another commonly used special stain in the histology lab.

What Does PAS Stain?

(1) Polysaccharides: The technique is commonly used to identify polysaccharides- these macromolecules are composed of monosaccharide units joined by covalent bonds. The main polysaccharide identified via histology staining in human and animal tissue sections is glycogen. This is present in numerous tissues, including skeletal muscle, cardiac muscle, liver, and kidney.

(2) Neutral mucus substances: It is also commonly used to stain and identify neutral mucus substances. These can include glycoproteins, glycolipids, and neutral mucins, which are produced by epithelial cells in different organs.

(3) Tissue basement membranes: These PAS-positive thin layers of reticular connective tissue anchor and support epithelium and endothelium to underlying connective tissue.

(4) Fungal organisms: The cell walls of some fungal organisms contain high levels of carbohydrate, and also stain PAS-positive. However, this only works on living fungi.

General Principles of the Stain

The reactivity of the PAS technique is based on the structure of the monosaccharide units.

The first reaction in the stain involves periodic acid acting as an oxidizing agent to oxidize the carbon-to-carbon bonds between two adjacent hydroxyl groups. This produces Schiff reactive aldehyde groups.

In the second reaction, the tissue section reacts with Schiff reagent. This comprises a mixture of basic fuchsin, hydrochloric acid, and sodium metabisulphite. The basic fuchsin in the mixture reacts with newly formed aldehyde groups in the tissue to produce a bright magenta color.

Finally, when the section is rinsed in water, bound fuchsin molecules in the tissue then produce a bright magenta color. The intensity of the color is proportional to the concentration of hydroxyl groups originally present in the monosaccharide units.

Haematoxylin is then typically used as a counter stain to visualize other tissue elements. When PAS is used to demonstrate fungal organisms, however, a light green counter stain is preferred.

Diastase (alpha-amylase) digestion may also be used to assist in the diagnosis of glycogen storage diseases. Diastase hydrolyses starch, glycogen, and breakdown products originating in these tissue polysaccharides. When compared to a slide of tissue containing glycogen, a diastase extraction slide will have no visible PAS stain.

Histologic feature	**Color with PAS Stain**
Glycogen, basement membranes, mucus substances, fungal organisms	Magenta
Nuclei	Blue (with haematoxylin counter stain)
Other tissue elements	Green (with light green counter stain)

Who Uses PAS Stain?

This stain is used widely for both diagnostic and research purposes. For example, researchers studying glycogen storage diseases or diseases of the basement membrane may routinely examine PAS-stained tissues sections to evaluate these respective elements in tissues of interest.

In diagnostic labs, pathologists often use this stain to help answer questions that may arise after examining routine H&E-stained sections. In particular, it can be used to help with the diagnosis of:

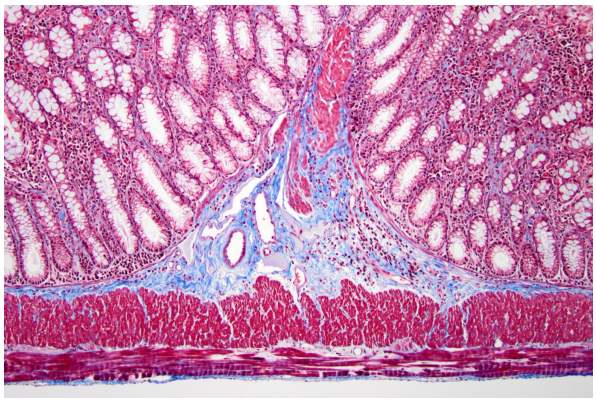
- Glycogen storage diseases: These are conditions in which excessive quantities of glycogen are stored in the liver, muscles, or kidney. PAS is often routinely used in the clinic to demonstrate glycogen accumulation in biopsies of these tissues.

- Tumors: Glycogen granules can also be present in some tumors, including some of those that arise in tissues such as the pancreas, lung, and bladder.

- Fungal infection: PAS can be used to visualize some fungal organisms in tissue sections.

- Basement membranes: Since PAS can be used to highlight the basement membranes of tissues, it can be used to identify disorders in which there is weakness or improper functioning of basement membranes – such as in the case of some glomerular diseases in the kidney.

Trichrome Stain for Collagen, Bone, Muscle and Fibrin



The trichrome stain is one of the most commonly used special stains in every histology lab.

The pedantic meaning of the word trichrome is 'three-colored', referring to the way in which the technique differentially stains tissue samples in three colors.

However, the term is now actually used to describe any staining method using two or more acid dyes of contrasting colors to selectively stain different basic tissue components.

What Does Trichrome Stain?

The stain uses three dyes to produce different coloration of different tissue types. Typically they are used to demonstrate collagen, often in contrast to smooth muscle, but can also be used to highlight fibrin in contrast to red blood cells. They can also selectively highlight other components.

Tissue Type	Color with trichrome stain
Collagen, Bone	Green-blue
Muscle, Fibrin, Cytoplasm,	Red
Red blood cells	Yellow or red
Nuclei	Dark red-black

Who Uses Trichrome Stains?

The stain is used widely for both research and diagnostic purposes. For example, researchers studying the progression of wound healing, or evaluating the efficacy of pharmacological products that accelerate it, will routinely examine trichrome-stained tissue sections to measure collagen deposition.

In diagnostic labs, pathologists often use this stain to help answer questions that may arise after examining routine H&E-stained sections. In particular, this stain is routinely requested to evaluate disease in the liver. It can be especially valuable when examining liver samples from patients with cirrhosis where it can provide helpful information about the degree of fibrosis, and therefore the stage and progression of their disease.

General Principles of the Stain

During the procedure, Bouin's solution (the 'mordant' which adheres dye to tissue) is applied to sections of tissue to intensify the final color. Nuclei are stained with iron haematoxylin. Cytoplasm and muscle are then stained red with Biebrich scarlet-acid fuchsin. Following treatment with the polyacids phosphotungstic acid (PTA) and phosphomolybdic acid (PMA), collagen is stained with trichrome blue, containing

aniline blue (or aniline light green which stains collagen green). Finally, an acetic acid solution is used to rinse the sections after staining which produces a more delicate shade of tissue coloration.

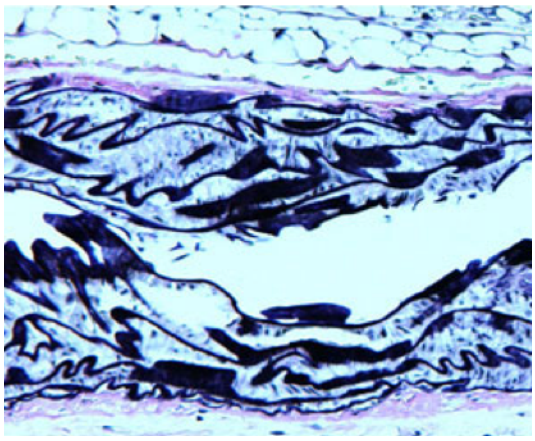
Different Trichrome Staining Techniques

Various trichrome recipes exist, all of which arose from pathologist Claude L. Pierre Masson's original formulation from the early 1900s. Of the various types of trichrome stains available, those most commonly used today are:

- Masson's Trichrome: This is a multi-step staining technique. It uses the polyacids PTA and PMA. All of the mordant and staining steps (as described above) are performed individually.

- Gomori's Trichrome: This is a one-step method staining technique. This method does not use polyacids, and unlike its Masson counterpart, it combines all reagents into a single solution (except for the nuclear stain and the Bouin's mordant) which is applied to tissue sections for a certain amount of time.

Verhoeff-van Gieson Stain for Elastic Fibers



Verhoeff-van Gieson (VVG) stain is the final special stain covered in detail in this eBook and is another stain used to identify connective tissue.

Ira Van Gieson first described the VVG staining protocol in 1889 as a method of evaluating collagen fibers in neural tissue. Frederick Herman Verhoeff, an American surgeon and pathologist, then modified the stain in 1908, as a method to differentiate collagen and other connective tissues, and highlight elastic fibers in particular.

What Does it Stain?

Elastic fibers are connective tissue fibers that allow tissues to stretch, and are abundant in the aorta where they provide flexibility to this large blood vessel. They are also present in other tissues that need flexibility, such as skin and lung.

These fine elastic fibers cannot typically be seen on routine H&E-stained tissue sections, therefore special stains are required to highlight them.

Although there are numerous special stains for identification of elastic fibers, VVG is most commonly used because it is quick, and produces intense staining of elastic fibers.

General Principles of the Stain

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:

> Verhoeff stain component: an iron-haematoxylin stain that is specific for elastic fibers. It forms strong bonds with elastin, the main component of elastic connective tissue.
>
> van Gieson stain component: a counterstain that is specific for collagen. It was named after American bacteriologist Ira van Gieson, and comprises two acid dyes- picric acid and acid fuchsin.

The technique involves staining the tissue specimen with a haematoxylin stain composed of ferric chloride and iodine, and comprises two main steps:

(1) Overstaining: this step involves a complex of haematoxylin–ferric chloride–iodine. The latter two components act as mordants to fix the haematoxylin stain to the tissues, and also act as oxidizing agents to help convert haematoxylin to haematein. The staining mechanism probably involves

hydrogen bond formation between the haematoxylin and tissue, although the specifics of this reaction are not well known. Since elastin has a strong affinity for the iron-haematoxylin complex in the stain, it holds the dye longer than other tissue components. Elastin therefore remains stained even after other tissue elements are decolorized.

(2) Differentiation: this step uses excess ferric chloride mordant to breakdown the tissue-mordant-dye complex. Sodium thiosulfate is used to remove excess iodine, and the van Gieson counterstain is used to produce contrast with the haematoxylin stain. The dye is attracted to the large volume of mordant in the differentiating solution and removed from the tissue. The elastic tissue, on the other hand, has the strongest affinity for the iron component of the stain.

Consequently, the end product of the reaction is such that elastic fibers and cell nuclei are stained black by the Verhoeff component. Collagen and muscle are stained red by the van Gieson counterstain, which also results in yellow staining of cell cytoplasm and other tissue components.

Who Uses Van Gieson Stain?

This stain is used widely for both diagnostic and research purposes.

In diagnostic labs, VVG is used to identify the presence or absence of elastic fibers in tissues. For instance, pathologists may use it to demonstrate loss of elastic tissue in the lung in patients with emphysema, and thinning and loss of elastic fibers in blood vessels of patients with arteriosclerosis.

Similarly, researchers investigating any of these types of diseases may also routinely examine VVG-stained tissue sections to detect the presence or absence of elastic fibers in tissues of interest. Other researchers may simply use the stain simply to differentiate connective tissue fibers in tissues of interest.

Images

All images used in this book are credited to Dr Nicola Parry, except the image for Congo Red stain taken by Ed Uthman (https://www.flickr.com/photos/euthman/3595031675/in/photolist-6tFtEK-6tFtDe-6tKBDm-6tFtDX).