Journal Homepage: - www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH (IJAR)

Article DOI: 10.21474/IJAR01/15542
DOI URL: http://dx.doi.org/10.21474/IJAR01/15542

*RESEARCH ARTICLE*

# SPECIAL STAINS AND IT'S APPLICATIONS IN HISTOPATHOLOGY

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*Manuscript Info*

***Manuscript History***
Received: 24 August 2022
Final Accepted: 27 September 2022
Published: October 2022

*Abstract*

Special stains are used to visualize various tissue elements and entities.They provide valuable information in the evaluation of various disease conditions.The principle on which they work is the interaction of intracellular and extracellular chemical reactions between the tissue components and the dyes.They allow the target substance to be identified on the basis of their chemical and biological character and thus help in diagnostic research.



## Introduction:-

**Staining** is the artificial colouration of the substance to facilitate it's examination by the use of a coloured organic molecule called dye.[1]

Histological staining is a series of technique processes undertaken in the preparation of sample tissues by staining using histological stains to aid in the microscope study. The process of histological staining takes five key stages which involve; fixation, processing, embedding, sectioning and staining .Staining is used to highlight important features of the tissue as well as to enhance the tissue contrast. Histological staining is commonly used for pathological diagnosis and in forensic studies.[2]

The most common and routine stain used in histopathology is a combination of haematoxylin and eosin **(H&E).** However, it simply cannot answer all the questions that a case poses at the plain diagnostic level, and it is clearly insufficient when one engages in an etiologic, histogenetic, or pathogenetic quest. As a consequence, the pathologists have always searched for additional techniques to probe those questions. Colloquially, these techniques have been referred to as "special", simply because they are applied only under special circumstances.[3]

Special stain is a term used to refer to many alternative staining techniques that are used when traditional H & E does not provide all the information the pathologist or researcher need from a tissue slide.

**Special stains** are "special" because they are not routine. They are applied to tissue sections in addition to hematoxylin and eosin (H&E)-stained sections to answer questions that arise above and beyond those that can be answered by interpreting H&E-stained tissue morphology. The term **"special stains**" is of uncertain provenance, but one can be certain that it began to be used after **1876** when H& E was introduced.[4]

Special stains belong to a diverse family of slide-based stains that rely on basic chemical reactions for microscopic visualization and general identification of various tissues, structures, cells, organelles, carbohydrates, minerals and microorganisms.[5]

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The aim of staining is to reveal the cellular and extra cellular matrix components and this forms the basis of histopathology. Stains highlight important features of the tissue as well as enhance the tissue contrast.Special stains are dyes that are used for special purpose and are not used routinely in a histopathology laboratory. They are of special interest in research and diagnostic.[3]

**Dyes** are coloured organic compounds able to selectively bind to tissue components. A **chromophore** is a part of the molecule capable of absorbing strong light at certain wavelength and transmitting or reflecting at others. Examples of chromophores are C=C, C=O, C=N, N=N, N=O, NO2. An important chromophore used in dyes is the quinoid arrangement. However, a compound can be coloured but still not stain because it lacks the ability to bind to tissues. To turn a coloured compound into a useful dye in histology, incorporation of an ionizable group that binds to tissue components is required.The ionizable group can be classified in acids, such as –COOH, -OH, -SO3H, and basic, that include primary, secondary and tertiary amines (-NH2, -NHR, -NR2). Usually, these functional groups are called **auxochromes.** They are attached to the chromophore modifying the ability of the chromophore to absorb light, altering the wavelength or the absorption intensity.[1]

The purpose of imparting colour / stain to certain components of the tissues is to assist in making diagnostic judgements.In physicochemical terms, staining occurs because a dye has an affinity for the tissue components. Affinity is itself dependent both on increasing entropy, with the overall system becoming more disordered, and on decreasing enthalpy, involving such phenomena as dye-tissue attractive forces. Dyes move from their solutions ("dyebaths") into the unstained tissues and cells because they are moving from regions of high dye concentration to regions containing less dye.[6]

Ionic bond or electrostatic interaction is the most important in histological staining and involves electrostatic attractions between opposite charges of dye and tissue.Another type is the covalent binding, this type of bond is the product of a reaction between the dye and the tissue and often a new C-C bond is formed. Covalent bonds are very strong and cannot be broken by procedures usually encountered in histological staining. Hydrogen bonds, Van der Waals, and hydrophobic interactions are included as well in the chemical dye interactions.[1]

Special stains however remain important tools for many pathologists, providing a powerful complement to immune histochemistry, flow cytometry, in situ hybridization and other diagnostic technologies that ultimately define a patient's medical profile. Many special stains are key to detecting and identifying pathogens, while others play an important role in diagnosing and monitoring cancer. Mucicarmine stains , for instance, demonstrate a poorly differentiated adenocarcinoma, Giemsa stains are useful in leukemia classification, and elastic stains gauge a tumor's degree of vascular invasion. Other clinical applications for special stains cover a wide range of diseases. Iron stains can indicate hemochromatosis or iron deficiency, the Masson's Trichrome stain demonstrates changes in collagen and muscle cells associated with cirrhosis, and the Periodic Acid-Schiff stainprovides information about glycogen storage disease and lupus-associated renal disease.[5]

**Classification**

**Table 1:-** Classification of special stains by Dr. Juan Rosai (2011), according to their use in laboratory [7]

1. Periodic acid Schiff ( PAS )
2. Organisms stains
3. Argentaffin and Argyrophillic stains,
4. Amyloid stains
5. Reticulin stains
6. Trichrome stains
7. Phosphotungstic acid hematoxylin
8. Stains for melanin,calcium and iron
9. Stains for neutral lipids
10. Mucin stains
11. Giemsa stains
12. Elastic stains
13. Myelin stains
14. Formaldehyde induced fluorescence

**Table 2:-** Classification of special stains according to the cellular components , modified and adapted by Veuthey T. Frontiers in Bioscience. 2014.

1. **Carbohydrates** : Alcian blue
   Periodic Acid Schiff (PAS)
   Mucicarmine
2. **Connective Tissue** :
   Collagen fibers : Masson's trichrome
   Mallory's trichrome
   Gomori'strichrome
   Elastic fibers: Verhoeff-Van Gieson
   Weigert'sResorcinFuchsin
   Orcein
   Reticular fibers :Wilder (silver impregnation)
   Gordon and sweet (silver impregnation)
3. **Lipids** : Sudan III and IV
   Sudan Black
   Oil red O
4. **Minerals**:
   Calcium :Von Kossa
   Alizarin red
   Copper :p -Dimethyl amino benzidine Rhodamine (DMABR)
5. **Nucleic acids** :Feulgen stain
   Ethyl green
   Pyronin Y
6. **Pigments**: Iron –Prussian blue
   Melanin-Masson Fontan ,Schmorl'sferricyanide
7. **Other Stains :**
   The Gram stain for bacteria
   The Grocott'sMethenamine Silver Stain for fungi
   The Warthin –Starry Stain for spirochetes
   The Giemsa stain for H.pylori
   Toluidine blue for mast cells.
   Von Kossa for bone.
   The Cresyl Violet Stain for identifying neurons

**Table 3:-** Working classification by Glick M. Burket's Oral Medicine. (2015).

**1.Stains for connective tissue**:

- -Van gieson's stain.
- Masson trichrome stain for muscles /PTAH OR phosoptungstic acid- hematoxylin for skeletal muscles.
- MSB technique for fibrin (Martius ,Scarlet,Blue )
- -Heidenhain's iron haematoxylin and Mallory's phospho tungstic acid haematoxylin for muscle striations
- Verhoeff, Orcein, Weigert'sresorcin-fuchsin and aldehyde fuchsin for elastic tissue fibres.
- Reticulin nuclear fast red stain for reticular fibres and silver stains (Gomori,Snook, Gordon &Sweets,etc).
- The Movatpentachrome method for demonstration of muscle, elastic fibres, collagen, ground substance and fibrinoid.

**2.Stains for protein :**

- Ninhydrin-Schiff method for amino groups.
- Millon reaction for tyrosine
- Performic acid –alcian blue method for disulphide and sulphydryl linkages.
- DMAB –nitrite method for tryptophan .
- Modified Sakaguchi reaction for arginin

**3.Stains for amyloid** -Congo red stain

**4 .Stains for carbohydrates**

- Periodic Acid –Schiff (PAS) to demonstrate the aldehyde groups
- PAS with Diastase to identify glycogen granules.
- Mucicarmine to stain mucin
- Alcian Blue for acid mucins and other acidic carbohydrate moieties.
- Alcian Blue /PAS for neutral and acid mucins.
- Colloidal iron for acid mucins

**5.Stains for lipids –**

- Oil red O
- Sudan black B

**6. Stains for pigments and minerals.**

For Pigments -

- The Fontana-Masson method & The Warthin-Starry method for melanin
- Mallory's and Perl's method for iron(hemosiderin)
- Oil red O,Sudan black B,Gomori's aldehyde fuchsin,Methyl green basophilia,Periodic acid Schiff and Fontana-Masson stain for lipofuscin.
- Van Gieson's solution for the bile in the liver &Gmelin technique or Stein's stain for bile pigments outside the liver.

For Minerals –

- Von Kossa& Alizarin red S for calcium
- Rhodanine dye for copper

**7.Stains for nucleic acid**

- Feulgen stain for DNA
- Methyl Green- Pyronin Y Stain for RNA

**8. Stains for microorganisms -**

- The Gram stain for bacteria
- Acid –Fast Bacteria Stain (AFB )Stain
- The Grocott'sMethenamine Silver Stain /Periodic Acid –Schiff-Green Stain for fungi
- The Warthin-Starry Stain for spirochetes.
- Alcian Yellow /toluidine blue (AY/TB) stain and Giemsa stain for H.pylori

**9.Stains for mast cells –**Toluidine blue

**10.Stains for bone -**

- Toluidine Blue O –for mineralized bone,osteoidseams,osteoblasts,osteoclasts.
- Alkaline Phosphatase –for osteoblasts.
- Tartrate Resistant Acid Phosphatase (TRAP)- for osteoclasts.
- Von Kossa –for demonstration of mineralization in bone.
- Goldner'sTrichrome –for differentiation of mineralized and non mineralized areas in bone

**11.Stains for neuropathology**

- The Cresyl Violet Stain for identifying neurons .
- Bielschowsky method for neurons and neurofibrils
- Phosphotungstic acid hematoxylin (PTAH),Holzer method and Cajal stain.
- Luxol fast blue(LFB)

**Table 4:-** Classification based on the stains most commonly used in routine Histopathology cases, ByMelanie Dobromylskyj.(2016).

**1.Metachromatic dyes**

- Giemsa: Blood smears, mast cells, infectious agents
- Toliduine Blue, Astra Blue : Mast cells
- Alcian blue/safranine : Different types of mast cell

**2. Infectious agents**

- Gram or Gram/Twort: Gram +/- bacteria
- Ziehl–Neelsen: Acid-fast bacteria, iron inclusion bodies
- Periodic acid-Schiff: Fungi (living)
- (+/- diastase digestion): Glycogen

- Grocott'smethenamine silver stain: Fungi (dead and alive)
- Warthin/Starry: Spirochetes
- Phloxine/tartrazine: Some viral inclusions
- Leishman: Blood cells, parasites
- Wrights:Leishmania
- Macchiavello:Rickettsiae, some viral inclusions
- Giemsa : Trypanosomes, Plasmodium, Chlamydia, Histoplasma

**3.Materials and tissue components**

- Congo Red, Sirius Red, Toliduine Blue: Amyloid
- Rubeanic acid, Rhodanine: Copper
- von Kossa, Alizarin red: Calcium
- Oil red O, Sudan III, Scharlach Red :Lipids, triglycerides, lipoproteins
- Alcian blue: Acid mucins (proteoglycans)
- Alcian blue/PAS: Acid and neutral mucins
- High iron diamine/alcian blue: Sulphated and carboxylatedmucins
- Massonstrichrome: Connective tissues
- Miller's Elastin
- Gordon & Sweets:Reticulin, liver structure
- Gomori hexamine silver: Basement membranes
- Haematoxylin van Gieson:Collagen, muscle
- Martius scarlet blue: Fibrin, connective tissue
- Safranine O/fast green: Cartilage
- Solochromecyanin: Myelin (non CNS)
- Luxol fast blue/cresyl violet: Myelin, nissl substance (CNS)
- Methyl green pyronin DNA, RNA

4.**Specific cells**

- Aldehyde fuchsin: Pancreatic islet cells
- Barrett's: Pituitary cells
- Carbolchromotrope:Eosinophils
- Grimelius:Argyrophil cells
- Naumenko/Feigin: Astrocytes

**5.Pigment stains**

- Masson/Fontana: Melanin
- Permanganate bleach: Removes melanin
- Dunn/Thompson: Haemoglobin
- Fouchet:: Bile pigments
- Perl's Prussian blue :Ferric iron
- Turnbull: Ferrous iron
- Schmorl:LipofusciN

**Special Stains For Carbohydrates**

Three of the most known stains for carbohydrates are :

1. Alcian blue
2. PAS
3. Mucicarmine

**Principle** :-

Alcian blue is a group of polyvalent basic dyes that are water soluble. The blue colour is due to the presence of copper in the molecule. The 3% acetic acid solution (pH2.5), Alcian blue stains both sulfated and carboxylated acid mucopolysaccharides and sulfated and carboxylated sialomucins (glycoproteins). It is believed to form salt linkages with the acid groups of acid mucopolysaccharides.[24-30]

**Interpretation** :-

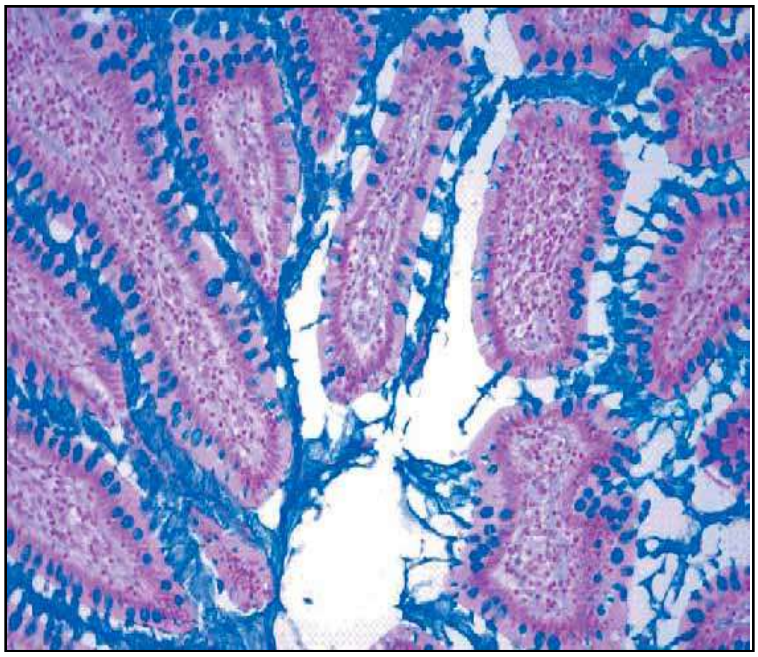


**Figure 1:-** Section of small intestine stained with Alcian blue to show mucus in goblet cells and in the spaces between the villi.

**Special Stains For Connective Tissue**

Connective tissue is one of the major types of tissue that connects the different parts of tissue and also supports the body parts. The connective tissue consists of two major elements:

**Cellular**-(1) Fibroblasts, (2) adipose cells, (3) macrophages, (4) pericytes and (5) mast cells

**Noncellular substances –**

The fibrous part of the connective tissue includes:

1. Collagen
2. Reticulin
3. Elastin
4. Basement membrane

**Collagen**

Collagen fibres are derived from the fibroblasts. Collagen fibre may remain as individual single fibre or in bunches. There are predominantly five types of collagen fibres.[39]

**Special Stains for Collagen**

**Masson Trichrome stains:-**

Many different colours of dye are used in Masson trichrome stain to differentiate the collagen fibres, muscle, fibrin and RBCs.

**Principle:**

In Masson trichrome stain, the dyes of different molecular weights are used in sequential manner.Acid fuchsin dye is used to stain both muscle and collagen tissue together. The staining time iskept for optimum duration so that the dye stains the muscle and collagen adequately.Thena polyacid of larger molecular size isused to differentiate the tissue. It removes the stain from the bone and collagen.Finally,another fibre-specific contrast dye(aniline blue) is used that has larger molecular weight than the previous (acid fuchsin) dye. This dye is kept for optimum time to stain the collagen fibres only.[40-41]

**Interpretation:-**

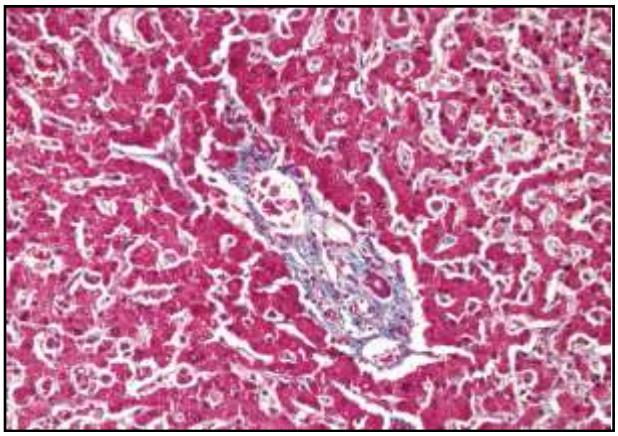


**Figure 2:-** Masson trichrome (MT) stain: the stain highlighting the portal tract bluish green, while the hepatocytes were stained red. The nuclei of the cells took a deeper shade of blue (normal liver tissue).

**Special Stains for Elastic Fibres**

**Elastic Fibres**

The common stains of elastic fibres include

1. Verhoeff's stain,
2. Orcein stain and
3. Weigert'sresorcin-fuchsin stain.

**Verhoeff's Stain for Elastic fibres**

**Principle:**

The haematoxylin dye binds with the elastic tissue by ionic interaction. Ferric salt acts as an oxidizer and helps in binding of haematoxylin and elastic fibres.[44]

**Interpretation:-**

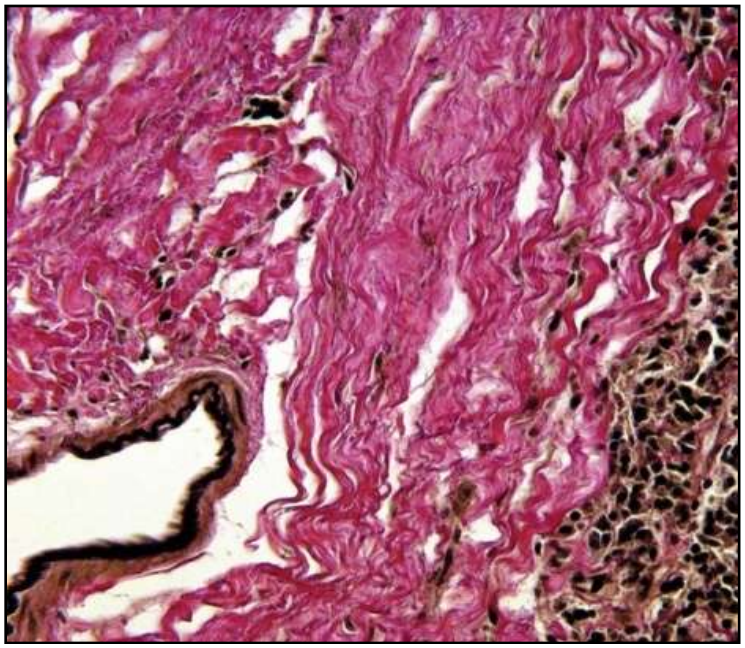


**Figure 3:-** Verhoeff–van Gieson stain showing loss of elastic fibers in upper and mid-dermis

**Special Stains For Reticulin Fibres**

Reticular fibers perform a support function in the body and are common in the liver, kidney and spleen.

**Principle**

Reticulin fibres are stained reliably by silver impregnation method. The reticulin fibres contain a carbohydrate component. Potassium permanganate is used to oxidize the reticulin fibres, and aldehyde group is generated from the carbohydrate component. The tissue is treated with silver salt in basic pH. In the basic medium, silver salt produces metallic silver that reacts with the aldehyde group of the tissue. Subsequently sodium thiosulphate is used to remove the excess unreactive silver. Tissue is further treated with gold chloride to make this precipitation permanent.[37]

**Interpretation**

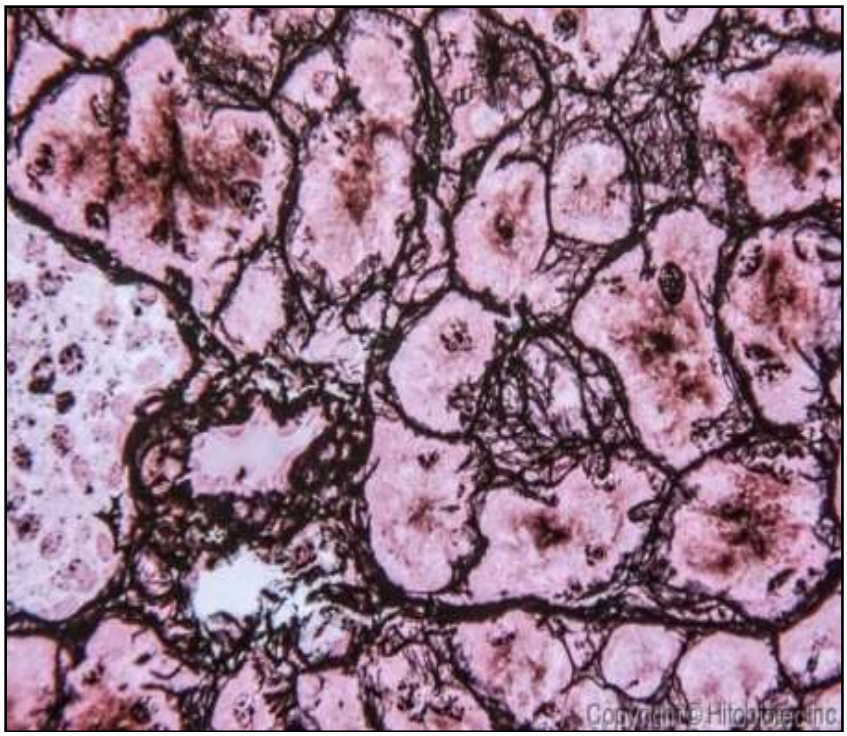


**Figure 4:-** Reticulin fibers in tissue section.

**Special Stain For Mast Cells**

Mast cells are found in the connective tissue and their cytoplasm contains granules (metachromatic) composed of heparin and histamine. Mast cells are identified in varied inflammatory conditions, particularly iskin. A toluidine blue stain is commonly used.[48-49]

**Toluidine Blue Stain**

Toluidine Blue is a small, weakly hydrophilic cationic dye. Attached to DNA or RNA , in chromatin or N is substance, this dye has a blue colour. Attached to GAGs, in mast cell granules or cartilage matrix, Toluidine Blue displays a purple "metachromatic" colour. Mast cells contain granules of histamine and heparin and are metachromatic. Reactionof these granules with toluidine blue causes them to stain a different colour than that ofthe dye solution. Skin is a good quality control tissue.[50-52]

**Interpretation**

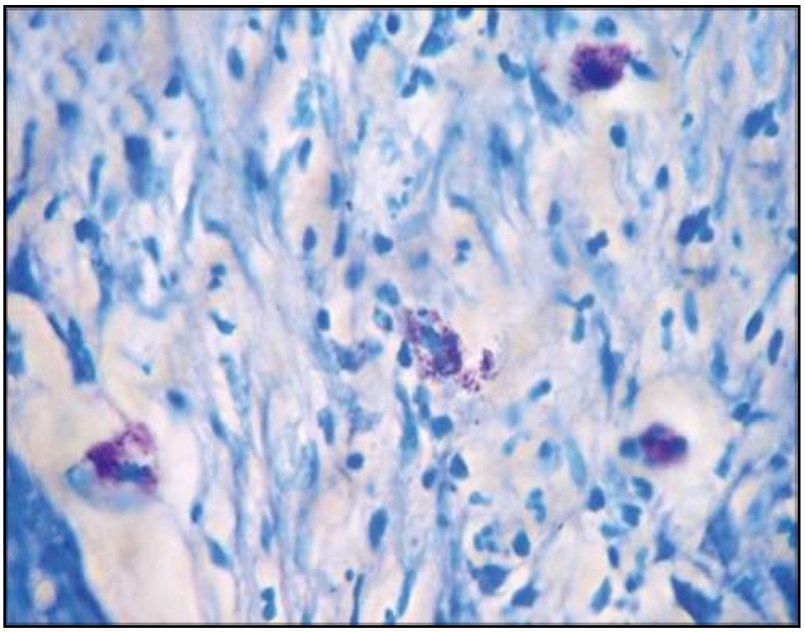


**Figure 5:-** Toludine blue staining mast cells in premalignant lesion.

**Special stains for lipids**.

1. Oil Red O
2. Sudan Black B

**Principle:**

Staining with oil-soluble dyes is based on the greater solubility of the dye in the lipid substances than in the usual hydro-alcoholic dye solvents.

**Interpretation:-**

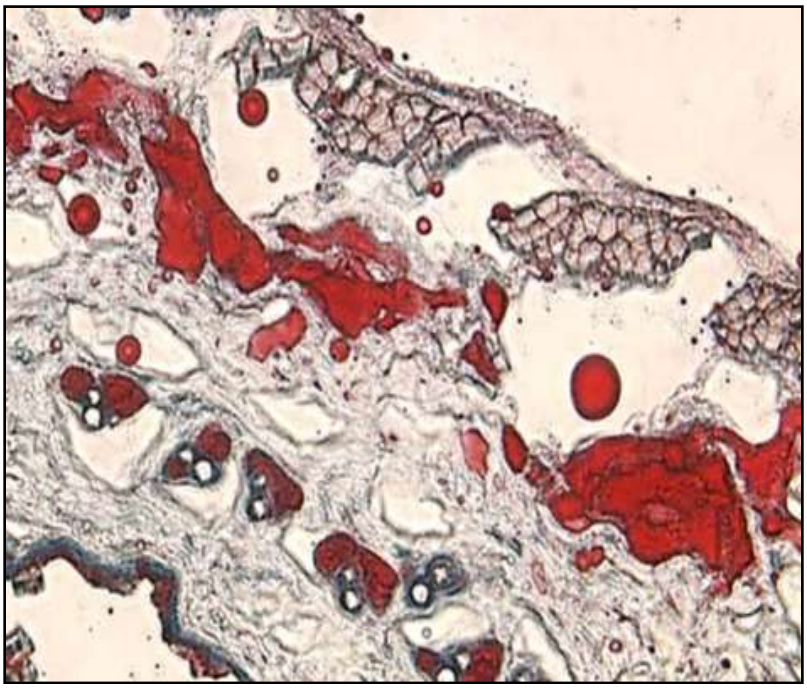


**Figure 6:-** Oil red O stain of fat emboli in lung.

**Special Stains For Minerals**
Minerals are important for the growth, development and function of the human body.

The most common minerals that can be demonstrated by special staining techniques are calcium, iron and copper.[60]

**Calcium**
Only calcium that is bound to an anion (such as PO4 or CO3) can be demonstrated. Calcium forms a blue-black lake with hematoxylin to give a blue color on H&E stain, usually with sharp edges.

Von Kossa stain is a silver reduction method that demonstrates phosphates and carbonates, but these are usually present along with calcium. This stain is most useful when large amounts are present, as in bone.[61-62]

Alizarin red S forms an orange-red lake with calcium at a pH of 4.2. It works best with small amounts of calcium (such as in Michaelis-Gutman bodies). The alizarin method is also used on the Dupont ACA analyzer to measure serum calcium photometrically.[66-67]

**Interpretation:**

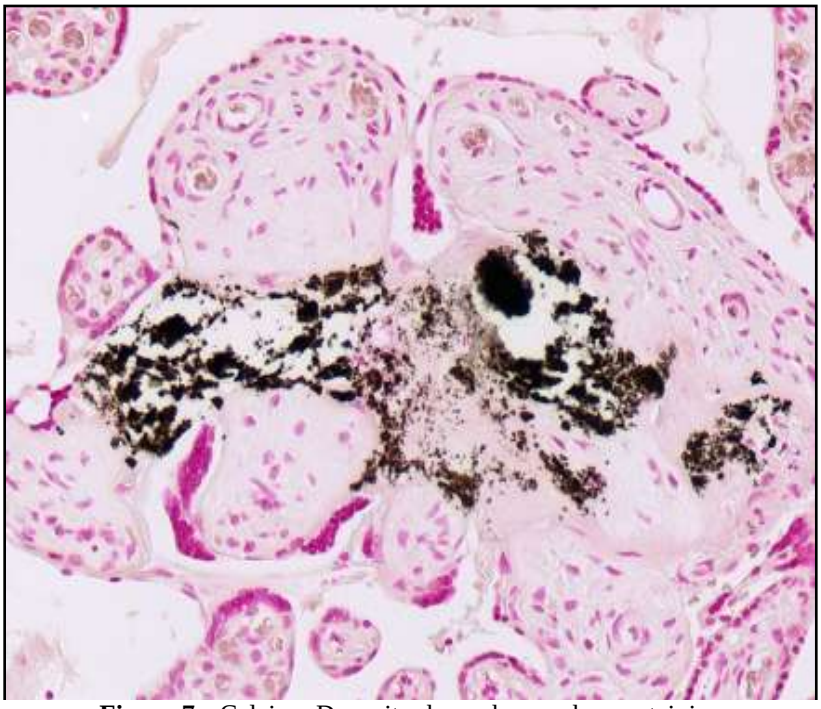


**Figure 7:-** Calcium Deposits shown by von kossa stainin.

**Special Stains For Nucleic Acids**
There are two types of nucleic acids, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). The two most common special staining techniques utilized for visualizing nucleic acids are the Feulgen and Methyl green-pyronin Y stains.[69-70]

**Feulgen Stain For DNA:-**
The Feulgen stain takes advantage of the ability of hydrochloric acid to hydrolyze or chemically alter the deoxyribose sugar of DNA into an aldehyde.

**Principle:-**
In this method, aldehyde groups produced by acid hydrolysis of DNA with HCl react with Schiff's reagent resulting in a purple staining. Because RNA is nothydrolyzed by HCl treatment, this technique is proper to selective DNA identification. [71-72]

**Interpretation:-**

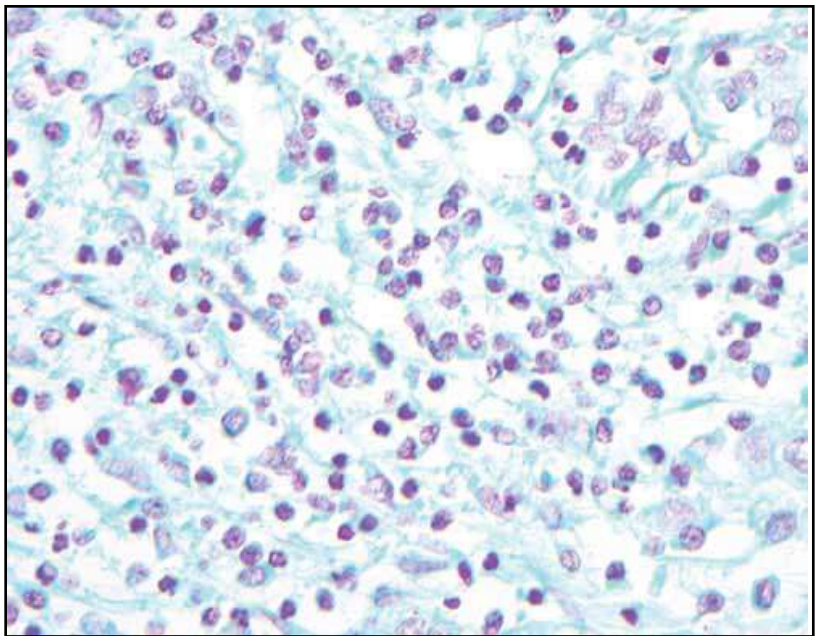


**Figure 8:-** Breast tissue stained with Feulgen

**Methyl Green-Pyronin Y Stain for RNA:-**

**Principle:-**

Methyl green is an impure dye containing methyl violet.When the methyl violet has been removed by washing with chloroform pure methyl green appears to be specific for DNA.While both dyes are cationic when used in combination methyl green binds preferentially and specifically to DNA, leaving the pyronin to bind to RNA.The methyl green-specific reactivity is attributed to the spatial alignment of the $NH_2$ groups of the dye to phosphate groups on the DNA double helix.[72]

**Interpretation**

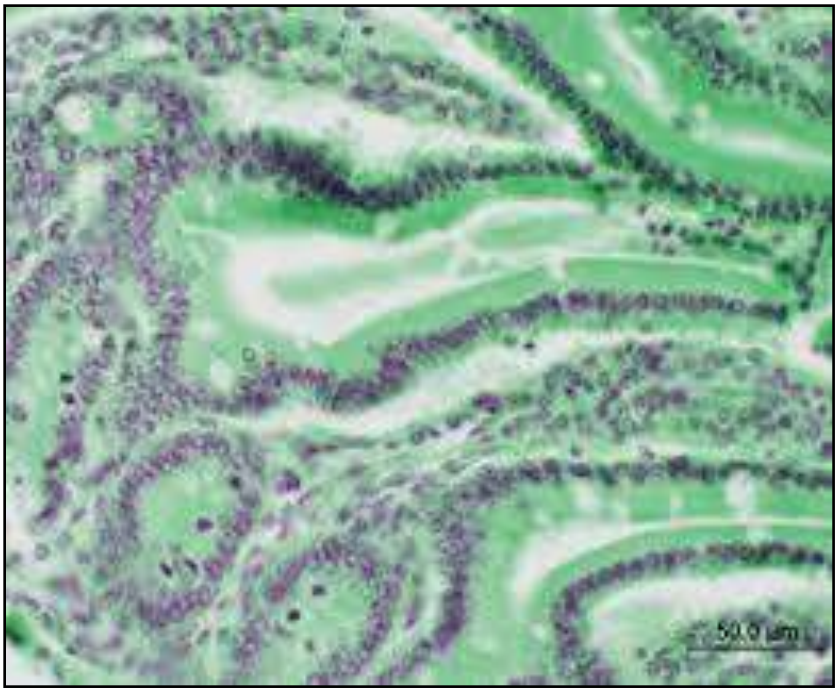


**Figure 8:-** Methyl green Pyronin for RNA.

**Special Stains For Pigments**
**Fontana-Masson ("melanin stain") method**
**Principle:-**
Masson's (1914) method (using Fontana's silver solution) and its various modifications, which also rely on melanin's argentaffin properties, are now widely used for routine purposes. Melanins are blackened by acid silver nitrate solutions. Melanin is also argyrophilic, meaning that melanin is coloured black by silver impregnation methods that use an extraneous reducer.

**Interpretation**

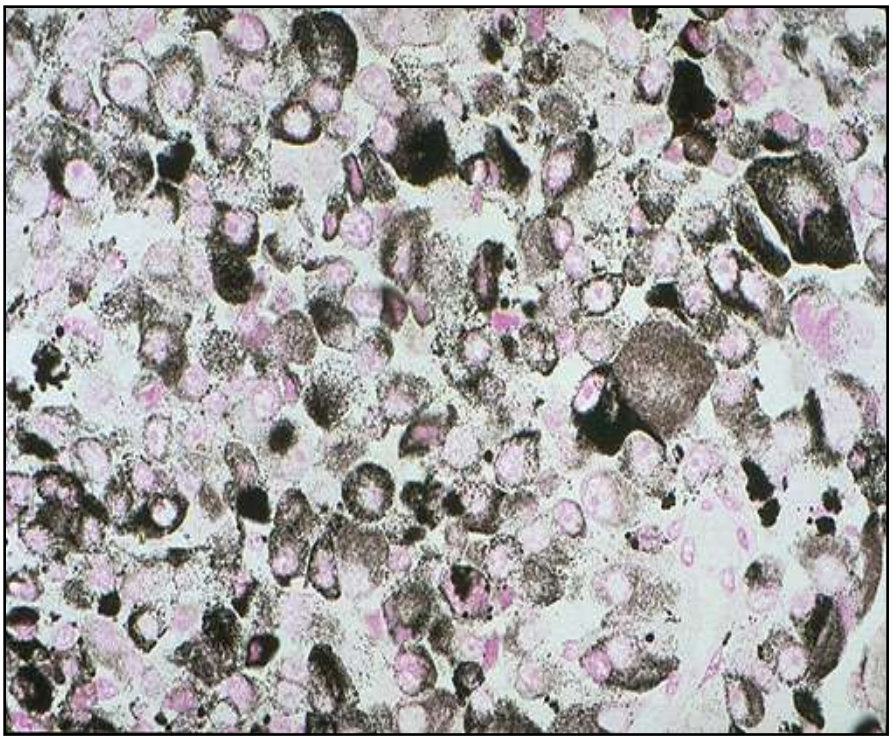


**Figure 9:-** Melanin pigment in cells of malignant melanoma, Fontana-Masson stain.

**Special Stains for Hemosiderin**
Hemosiderin, a blood-derived pigment, is a crystalline aggregate of proteins involved in iron storage. Some of the pathological conditions involving hemosiderin are hemorrhage, hemolyticanemia, metabolic iron disturbances, liver fibrosis, toxins, cirrhosis, heart failure and diabetes mellitus.

**Prussian blue**
**Principle:-**
Prussian blue reaction involves the treatment of sections with acid solutions of ferrocyanides. Any ferric ion (+3) present in the tissue combines with the ferrocyanide and results in the formation of a bright blue pigment called Prussian blue, or ferric ferrocyanide. This is one of the most sensitive histochemical tests and will demonstrate even single granules of iron in blood cells.[75-76]

**Interpretation**

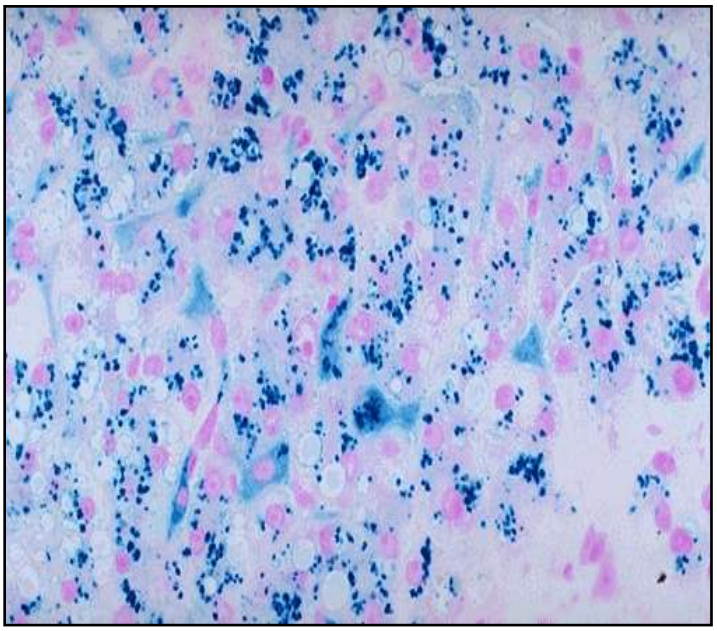


**Figure 10:-** Hemosiderin, liver, Iron stain.

**Special Stain For Proteins**

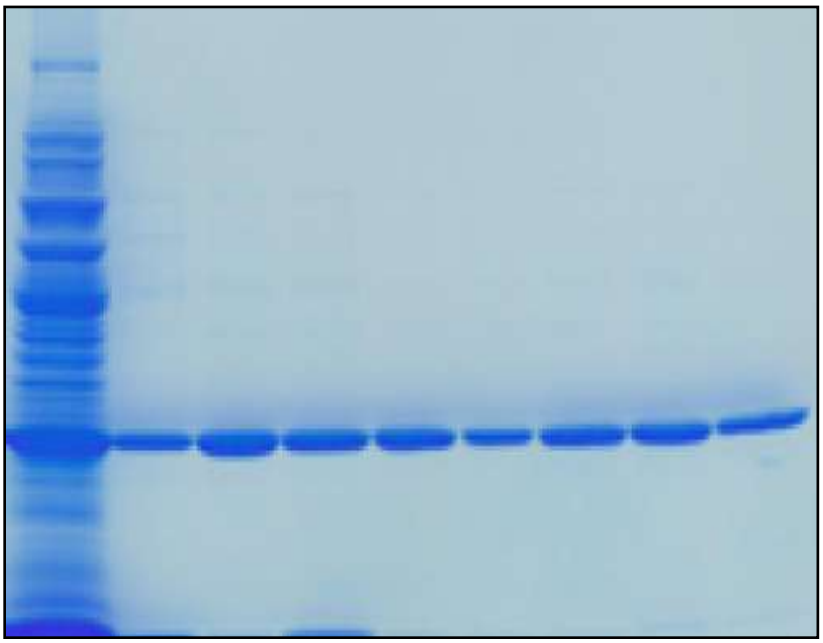
**Coomassie Blue Stain**

The most common method of in-gel protein detection is staining with Coomassie dye. Colloidal Coomassie can be formulated to effectively stain proteins within 1 hour and requires only water for destaining.

**Principle**:-

In acidic conditions, Coomassie dye binds to basic and hydrophobic residues of proteins, changing in color from a dull reddish-brown to intense blue. Coomassie staining can detect as little as 8–10 mg per band for some proteins and 25 mg per band for most proteins.

**Interpretation**



**Special Stain For Micro-Organisms**
**Gram staining method for Bacteria**
Gram staining differentiate bacteria into 2 classes depending on their cell wall structure and composition

**- Gram positive**
**- Gram negative**

1. Crystal violet is first applied to the section as a primary stain, followed by iodine mordant forming a dye lake. The section are then decolorized using alcohol / acetone mixture
2. Gram positive cell do not decolorize at this step and gram negative bacteria will not retain crystal violet

**Principle:**

1. Differential Staining.
2. Gram positive cell wall contain thick layer of peptidoglycan with numerous teichoic acid cross linking which resists the decolorization.
3. In aqueous solutions crystal violet dissociates into CV+ and CV – ions that penetrate through the wall and membrane of both gram-positive and gram-negative cells. The CV+ interacts with negatively charged components of bacterial cells, staining the cells purple.
4. When added, iodine interacts with CV+ to form large Crystal violet iodine (CV-I) complexes within the cytoplasm and outer layers of the cell.
5. The decolorizing agent (ethanol /ethanol and acetone solution), interacts with the lipids of the membranes of both gram positive & gram negative Bacteria.
6. The outer membrane of the gram negative cell (lipopolysaccharide layer) is lost leaving the peptidoglycan layer exposed.
7. Gram negative cell walls become leaky & allow the large CV-I complexes to be washed from the cell.
8. The highly cross-linked and multi-layered peptidoglycan of the gram positive cell is dehydrated by the addition of ethanol.
9. The multi-layered nature of the peptidoglycan along with the dehydration from the ethanol treatment traps the large CV-I complexes within the cell.
10. After decolorization, the gram+ve cell remains purple in colour, whereas the gram-ve cell loses the purple colour and is only revealed when the counterstain, the positively charged dye safranin, is added.
11. At the completion of the Gram stain the gram+ve cell is purple and the gram-ve cell is pink to red.[84]

**Interpretation:**

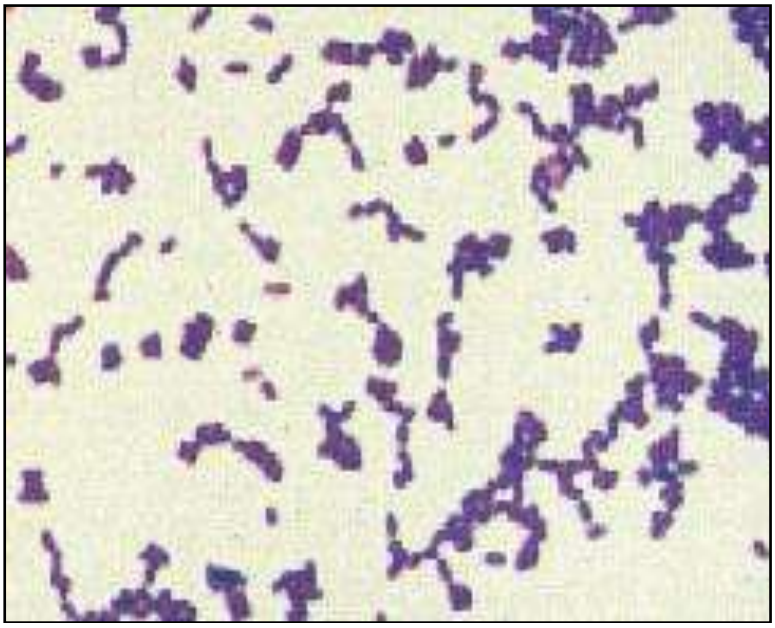


**Figure 11:-** .a Gram positive.

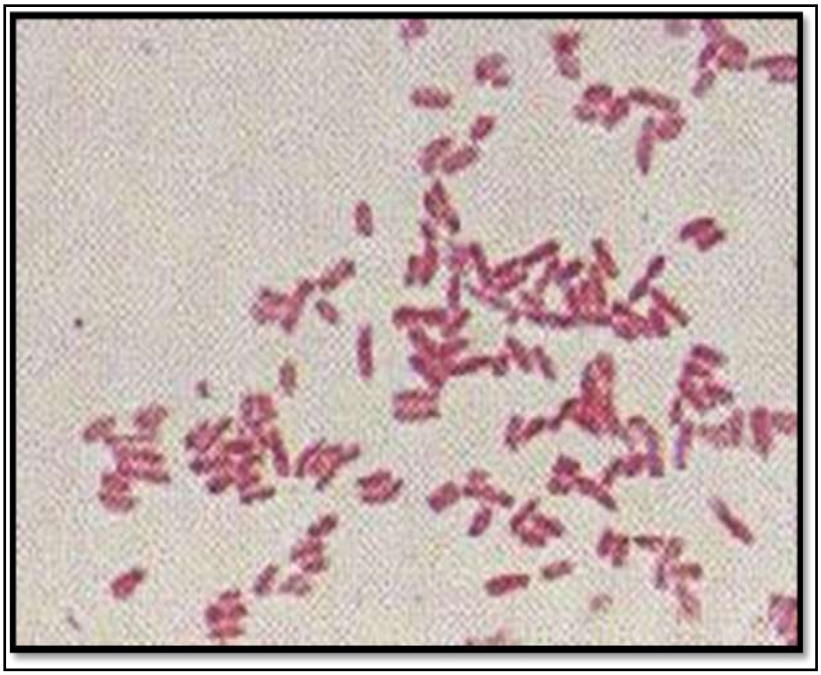


**Figure 11:-** b Gram negative.

**Summary**

The significant development of the science in the last decades increased the need and the interest to know cellular and structural details, and it was possible due to the microscopic identification of cells and their components, being improved even more with the development of histological techniques. A histological staining is basically the procedure of staining some cellular structures by using dyes, which besides providing colour must be capable of binding to certain tissue components but not to all of them.

In this review, the main features of staining mechanisms and the relevant properties of dyes for its use in histology were outlined. In addition, some of the applications performed in the last decades were described. Because there are many histological techniques for the detection of several tissue components and it would be impossible to do a complete revision, the attention was centered in the most commonly used stains.

Taking into account advantages and disadvantages, histological stains continue to be a unique tool, due to its simplicity and reproducibility providing valuable information about cell structures and tissue morphology and pathology, thus contributing to several research fields.

We can be proud that basic histological, histochemical and immuohistochemical methods have a very long and productive history and continue to give us useful information. We have an enormous debt to the pioneers who discovered stains for colouring tissues and combating disease together with subsequent techniques. These old methodologies still continue to play an important role in the histopathology laboratory and remain at the forefront of research in these disciplines with an enormous long-term impact in cell biology and molecular biology.

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