A 'Dyeing' Art in Microbiology

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Methodologies, practices, techniques and systems continue to change, evolve and develop in the scientific community. Throughout all of this there are often constants – spare a thought for the simple stain, a foundation in diagnostic cellular identification.

There are approximately 4500 published formulations for stains, and countless variations, not least due to personal changes, and even mistakes. From a simple colour point of view there are over 27000 (*CI) recognised colours with set wavelengths, and again countless variations, mainly due to technique.

Staining methods have long been used as an essential aid to diagnosis of disease, cellular differentiation and bacterial identification, and still have an irreplaceable role in many disciplines in hospital diagnostic pathology departments. Stains will be found in routine use in cytology, histology, haematology, microbiology and parasitology, for studying a range of specimens in both in-vitro and in-vivo conditions.

As we now move further into the molecular and electronic age, will we always need the stain?

Will multinational corporations fade away, will the Microscope only be seen in museums, will the biomedical scientist's skills be even further eroded and valued less? Anyone can push a button and call a help line. Only skilled biomedical scientists can interpret a stain and use the technique.

Where did it all begin?

Ask a tourist what Delft is famous for, and it will more than likely be delicate blue collectable pottery, rather than Microscopes and staining technique origins.

Some of the most important origins of staining date back not to a scientist, but to a Dutch draper known as Antonie Van Leeuwenhoek (1632 – 1734), whose hobby was the grinding of glass, which he used to examine threads in fabrics and later a variety of biological samples. Born Thonis Philipszoon, he became known as Van Leeuwenhoek perhaps as he was born in a house on the corner of Lions Gate in Delft, Van Leeuwenhoek translating literally as *From Lions Gate*. (*Figure 1*)

Figure 1. The famous Lions Gate

Figure 2. A representation of Van Leeuwenhoek's first microscope

Due to his skills in grinding glass lenses which at the time were no more than slivers of glass, Van Leeuwenhoek is today credited with the discovery of the microscope, and further to this was the first to observe blood cells and bacteria using this simple invention. He was the only one who made these observations and it would take many years before other people could confirm them. *Figure 2* shows a representation of this first microscope, a far cry from the advanced systems available today.

The 17th and 18th centuries saw many advances in science, and competition between scientists was fierce as they strived for discoveries and acknowledgement. Van Leeuwenhoek avoided confrontation and carried on his work quietly. Had it not been for a learned friend Dr Regnerus de Graaf, he may never have been credited with his discovery, it being de Graaf who sent the first transcripts of his work to the Royal Society of London.

Figure 3. The compound microscope

Founded in 1660, The Royal Society, possibly the oldest scientific society, had many now legendary scientists and inventors as founder members including Robert Boyle, Christopher Wren, Isaac Newton and Robert Hooke.

It was Robert Hooke who then furthered the development of the microscope and is one of a number of scientists credited with the invention of the compound microscope (*Figure 3*) using more than one lens and allowing magnification up to x1000 for the first time.

Now that we had microscopes, we needed colours to differentiate cells. Working alone in Delft in 1714 Van Leeuwenhoek was examining the muscles of fat and lean cows, and found that the material was too transparent for clear observation. It is here that his work on the theory of staining began. He treated the muscle with an extract obtained by macerating Saffron in burnt wine. The Saffron, taken from the styles and stigmas of the *crocus sativus* flower, native to Spain, is still used to this day as a stain in Histology to show tissue structure. This method opened the door for many naturally occurring dyes to be experimented with for staining all manner of cells, structures and tissues.

In 1849, Goppert and Cohen, used Carmine with limited success, and in 1854 Hartig continued the work with Litmus, Black Ink and Copper Sulphate and is credited as a front runner in the process of Histological staining, later completed by Gerlach who actually made his most important discovery by accident that lead to a huge advance with the staining of nerve fibres and nerve cells using Amnoniacal Carmine. This spurred many researchers on, and today there are hundreds of differential staining techniques using numerous blends of pigments and dyes. These can be referenced in the International Colour Index first published in 1925 containing over 27,000 individual products offering a valuable reference for the scientific, textile, paint, printing and plastic industries. Pigments and dyes can be acidic, basic, dispersing, direct, fluorescent, mordant and reactive, and can be used in many staining techniques such as direct, simple, indirect, progressive, regressive, vital, intra-vital, supra-vital, negative, impregnative, polychromatic and metachromatic.

A Common Example....

Perhaps one of the most widely used staining methods in Microbiology is the Gram Stain, and although one of the simplest staining methods used, it is one of the most fascinating polychromatic methods enabling initial differentiation based on colour and morphology.

Gram staining is named after the Danish microbiologist Hans Christian Gram, who initially researched the method in 1882 and published the first recommended method in 1884. It is still one of the most important staining techniques in microbiology being one of the first tests performed for the identification of bacteria offering valuable information for clinical diagnostics.

The principle stain of the Gram's Method is crystal violet, sometimes substituted with methyl violet. The method allows for a crystal violet-iodine complex to form in the protoplast of all organisms stained. After decolorizing, those organisms that are able to retain this dye complex are classified as Gram positive; organisms that are decolorized and take up the counterstain are classified as Gram negative.

The Gram staining method is based on the ability of a bacterial cell wall to retain the crystal violet or methyl violet during solvent treatment. The cell wall of Gram positive microorganisms has a higher peptidoglycan and lower lipid content than Gram negative bacteria. Once the bacterial cell

wall is stained, Iodine is used as a mordant to form the crystal violet-iodine complex so that the dye cannot be easily removed. The use of this mordant, derived from the Latin word to bite, *Mordere*, is an essential step in the method to distinguish Gram positive and Gram negative microorganisms.

Subsequent treatment with a decolourizer or differentiator dissolves the lipid layer from the gram-negative cells. This removal of the lipid layer enhances the leaching of the primary stain from the cells into the surrounding solvent. Also the solvent dehydrates the thicker Gram positive cell walls, closing the pores as the cell wall shrinks during dehydration. This effectively blocks the diffusion of the violet-iodine complex, and the microorganisms remain stained. The length of the decolourization is critical in differentiating the Gram positive microorganisms from the Gram negative microorganisms. Prolonged exposure to the decolorizing agent will remove all the stain from both types of microorganisms.

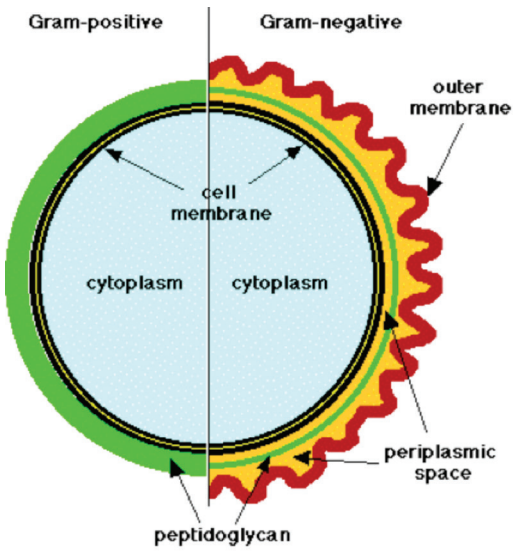

Figure 4. The basic structure of the Gram Negative and Gram Positive cell wall

Finally, a counterstain such as safranin, neutral red, dilute carbol fuchsin or basic fuchsin is applied to give decolorized Gram negative bacteria a pink/red colour. The selection of the counterstain can be personal preference or application driven. Safranin and Neutral Red offer very similar results, dilute carbol fuchsin and basic fuchsin stains many Gram negative bacteria more intensely, making them easier to see. *Figure 4* show the basic structure of the Gram Negative and Gram Positive cell wall.

Microorganisms which are poorly stained by safranin, such as *Haemophilus spp.*, *Legionella spp.*, and some anaerobic bacteria, are better stained by fuchsin counterstains rather than safranin.

Care must always be taken, Gram Staining is an art form, practiced over many years by all microbiologists, and results can vary if care is not taken.

Recommended method for Gram Staining

1. Prepare a thin, uniform smear of specimen and air dry.
2. Heat fix and allow to cool.
3. Flood the slide with Crystal Violet or Methyl Violet, stand for 1 minute. Rinse with water.
4. Flood the slide with Iodine, stand for 1 minute. Rinse with water.
5. Gently decolorize with Differentiator for approximately 10 seconds. Rinse with water.
6. Flood the slide with counterstain, stand for 30 – 60 seconds.
7. Rinse well with water, gently blot dry.
8. View using oil immersion microscopy.

Quality control is important in any laboratory method, and this also applies to staining. The age of the cultures and the pH of the medium in which the bacteria are grown can markedly affect their reaction to the Gram stain. It is recommended to use fresh cultures up to 24 hours old for quality control of the Gram staining method.

Recommended QC cultures; *Escherichia coli* NCTC 10418 (Pink to Red Gram Negative Bacilli), Oxford *Staphylococcus aureus* NCTC 6571 (Blue to Purple Gram Positive Cocci), Haemolytic *Streptococcus* Group A NCTC 8198 (Blue to Purple Gram Positive Cocci)

Around the same time that Gram introduced the Gram Stain, the acid fastness of some bacteria was noted by Paul Ehrlich in 1882, and a stain for Acid / Alcohol Fast Bacilli (AFB), primarily for Mycobacterium species was introduced by Ziehl – Neelsen in 1883, the ZN stain. This then became the classic method for staining *Mycobactrium tuberculosis*.This method requires heat to be applied to the specimen slide, although a "cold" alternative is available with the Kinyoun staining method.

Fluorescence microscopy was introduced early in the 20th century and initially observations were limited to specimens that fluoresce naturally, then fluorescent dyes for staining tissues and cells were investigated. During the 1940s fluorescence microscopy became popular, when Coons and Kaplan introduced a technique to label antibodies with a fluorescent dye to study antibody-antigen interactions. This lead to major changes and important developments in the field of immune histochemistry. Then in 1994, the discovery that really brought fluorescence microscopy to the forefront was when M. Chalfie et al. succeeded in expressing a naturally green fluorescent protein (GFP), in living organisms, leading to additional research for a whole new class of tagging methods.

The development of fluorescent microscopy also lead to the Auramine Phenol Stain becoming a popular choice for screening sample smears for AFB's, although all positives still require checking with the traditional ZN staining method.

In 1931, Ernst Ruska and Maximilian Knoll were credited with the invention of the Electron Microscope and by 1964 resolution of 1nm was possible.

1590-1610	Initial records of the Microscope with Hans and Zacharias Janssen
1652-1720	Development of the Van Leeuwenhoek Microscope and first staining techniques with Saffron.
1660	The Royal Society of London founded in London.
1673	Van Leeuwenhoek work taken to the Royal Society.
1729	Achromatic lenses for spectacles were developed by Chester Moore Hall.
1807	Achromatic lenses made commercially by Harmanus van Deyl.
1849-1854	Early tissue staining methods with Carmine, a naturally occurring dye by Goppart, Cohen and Hartig.
1856	Introduction of the use of Analine dyes.
1858	Initial staining techniques developed for nerve cells by Gerlach.
1880	First oil immersion objective lens introduced by Carl Zeiss Jena.
1882	Acid fastness described by Erhlich.
1883	Introduction of the AFB staining technique by Zeilh-Neelsen.
1884	Introduction of the Gram stain by Hans Christian Gram.
1904	Principles of fluorescent microscopy seen by Kohler.
1931	Ernst Ruska and Maximilian Knoll were credited with the invention of the Electron Microscope.
1934	Phase contract Microscope developed by Zernike.
1964	Resolution of 1nm was possible with Electron Microscopy.
1994	M. Chalfie et al. succeed in expressing a naturally fluorescent protein, the now-famous green fluorescent protein (GFP), in living organisms, a major breakthrough for fluorescent microscopy.
2007	Pro-Lab Diagnostics revolutionise staining with the introduction of the Poly Stainer in Microbiology.
2011	New regulations tighten the production and quality control of staining solutions.
2015	Yet more changes to the regulations with the introduction of the CLP EC Regulation on Classification, Labelling and Packaging.
Present Day	Stains are still with us and being used extensively.

Traditional Hands on or Automation?

As workloads increase in busy laboratories we are presented with many challenges for more accurate results in a more timely fashion, and this inevitably leads to the question of automation. A time span of almost 360 years has seen major advances in Microscopy from its humble beginnings with the Van Leeuwenhoek Microscope, to the scanning electron microscope. Throughout this time, many staining techniques have been developed and tradition remains with some of the most tried and trusted methods still used today as mentioned above.

Many staining methods can, and have been automated offering accurate and consistent results, whilst freeing up valuable laboratory technician time. This automation can be achieved in all disciplines, and in Microbiology the Poly Stainer, manufactured by IUL Spain and available from Pro-Lab Diagnostics in the UK, offers the ultimate in automated staining for a variety of Microbiological staining methods, for example – Gram staining, TB staining, Cryptosporidium staining and Trichomonas staining.

Watch your Oil!

With the development of the first oil immersion lens in 1880 by Carl Zeiss Jena, the use of immersion oil was also required. Magnification was improved with the use of oil immersion lenses and the image quality could be directly related to the optical properties of the immersion oil used. Therefore, the use of oil with the same refractive index as the glass used in the manufacture of glass microscopy slides, increases the resolution between the oil immersion lens and the slide by replacing the air and easing the spread of light at the same speed as in glass (1.513), hence avoiding image distortion.

Hazards can be associated with certain oils, and the correct choice is important. Preferably oils free of hazards such as Dibutyl Phthalate (DBP) should be used. (Ref - PL396 Immersion Oil from Pro-Lab Diagnostics).

Check your Compliance

The manufacture of staining solutions with consistent high quality and guaranteed performance is only part of the responsibility taken by Pro-Lab Diagnostics, a leader in this field. Compliance with regulations is also essential to meet safety requirements, strict labelling requirements, CE marking, compliance with the InVitro Diagnostics Directive, COSHH regulations and the new R.E.A.C.H (Registration, Evaluation, Authorisation and restriction of Chemicals).

Full details can be obtained from Pro-Lab Diagnostics with the 'Stain Connection' service offered for Microbiology Stains, recommended methods and automated techniques.