



DYEING TO GET IT RIGHT

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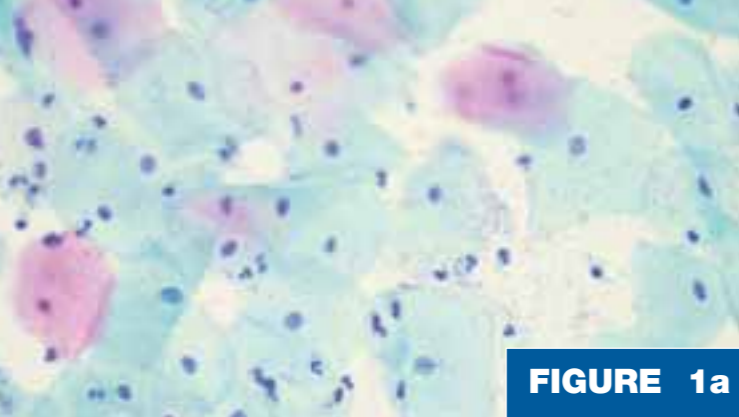


FIGURE 1a

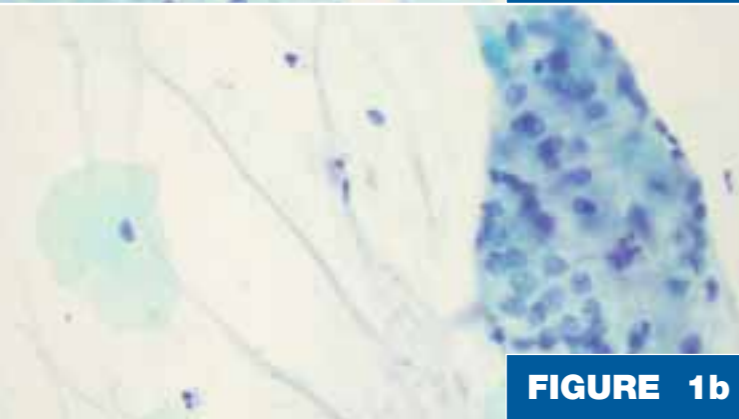


FIGURE 1b

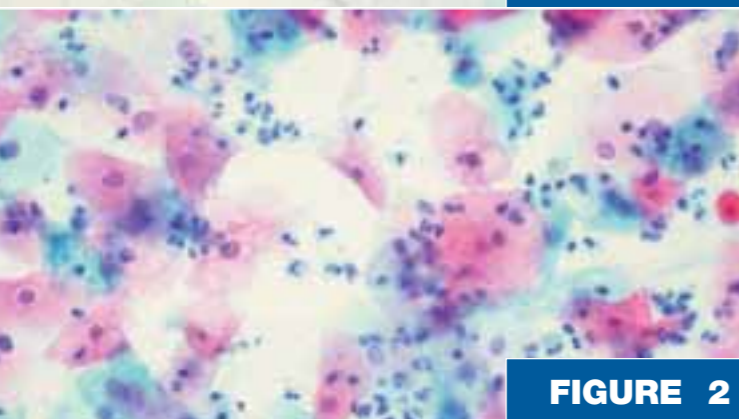
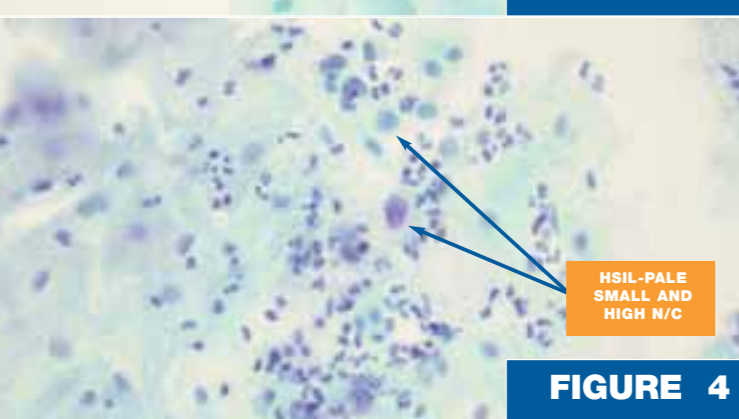


FIGURE 2



FIGURE 3



HSIL-PALE SMALL AND HIGH N/C

FIGURE 4

ABSTRACT

The universal and most popular method of staining Pap smears is through the 'Pap-stain' method, named after its developer, Dr. George N. Papanicolaou. Developed in the late 1940s, and modified by him in 1954 and 1960, it is a polychrome staining reaction designed to demonstrate variation of cellular maturity and metabolic activity.

Accurate cytological assessment may not be possible unless the smear is optimally fixed and stained. A well-stained Pap smear should demonstrate crisp blue/purple nuclei. Cytoplasmic staining should show a broad spectrum of colours ranging from orange in highly keratinised cells through ranges of orange/pink in superficial cells and turquoise green/blue in intermediate and parabasal cells. Poor staining often results in nuclear abnormalities being misgraded or sometimes completely missed.

The poster is an overview of the stain method, the dyes used, assessment of the stain, troubleshooting and safety aspects.

INTRODUCTION

The Papanicolaou stain was developed by Dr. George N. Papanicolaou in 1942, and subsequently modified by him in 1954 and 1960. It is a polychrome staining reaction designed to demonstrate variations of cellular maturity and metabolic activity.

There are four main steps in the staining procedure:

- (1) Fixation.
- (2) Nuclear staining.
- (3) Cytoplasmic staining.
- (4) Clearing.

The cell samples are smears and must be immediately fixed in 95% ethanol or a spray fixative that contains polyethylene glycol.

DYES

The Papanicolaou stain is comprised of a nuclear stain, hematoxylin, and three cytoplasmic dyes: orange G, eosin Y, and light green. Bismarck brown, a basic dye, was present in the original Papanicolaou stain but is no longer used in many preparations. The nucleus is stained first to demonstrate the chromatin material within, and then the cytoplasm is stained to provide contrast. Hematoxylin is used to demonstrate nuclear detail. The procedure can be performed using a progressive or a regressive method. In progressive staining, the reaction is stopped once the desired staining intensity has been achieved. In regressive staining, the smears are overstained and then differentiated in a dilute aqueous or ethanol and hydrochloric acid solution to achieve the desired results. The nuclear stain shifts from blue/purple to a pink/red. After differentiation the smears are rinsed in water and placed in an alkaline "bluing" solution to re-establish the insoluble blue coloured dye lake. Bluing solutions include Scott's Tap water, alcohol ammonia and lithium carbonate. A thorough rinse is necessary after this step. Many laboratories tend to use the progressive method of staining to avoid over/under differentiating and because it is easier to maintain standardization from one stain batch to another. The decision to use a progressive or regressive method lies solely with the personal preference of the user.

The first cytoplasmic stain is Orange G. It is prepared in an ethanol solution with phosphotungstic acid. It stains cytoplasm yellow to orange if keratin or prekeratin is present.

The second cytoplasmic stain is EA and it consists of a mixture of light green and eosin Y in an ethanol solution with phosphotungstic acid. Eosin should stain superficial squamous cells, nucleoli, cilia and RBCs. Light green should stain the cytoplasm of metabolically active cells, such as metaplastic and intermediate squamous cells.

DEHYDRATING AND CLEARING AGENTS

The most common dehydrating agent used in the cytology lab is ethanol. Ethanol is a clear, colourless, flammable liquid. It is strictly controlled by the federal government and extensive record keeping is required. Ethanol is a rapid and efficient dehydrant.

Clearing originally received its name because many of the reagents used for this purpose have a high refractive index and render the exposed specimen transparent. This is particularly useful when viewing stained specimens microscopically, since it is advantageous to have a consistent refractive index for the specimen, mounting medium, glass slide and coverslip.

Xylene has been the most widely used clearing agent for many years. It is an aromatic hydrocarbon that rapidly replaces ethanol and has a refractive index capable of rendering the tissue transparent. It turns cloudy in the presence of water. It is a flammable reagent that should only be used with adequate ventilation, and skin contact should be avoided. The use of nitrile gloves is useful in the handling of this chemical. These more stringent requirements for occupational safety and environmental protection have driven the replacement of xylene with more user-friendly alternatives which are free of aromatic hydrocarbons. There are two types of xylene substitutes gaining popularity; limonene reagents, which are derived from citrus fruits and short chain aliphatic hydrocarbons, which have a low toxicity and are considered to be non-irritating and non-sensitizing.

THE GOOD, THE BAD AND THE UGLY OF STAINED PAP SMEARS

Hematoxylin staining of individual nuclei should be clearly visible at low power (10x objective), and blue to purple in colour. At high power (40x objective), nuclear chromatin should be clearly demonstrated and appear granular, crisp and distinct.

There should be no back ground staining, apart from cervical mucus, and Hematoxylin should not adversely affect the colours of the counterstains (see Fig 1a, 1b). When assessing the quality of the Pap stain, smears with Candida infection and bacterial vaginosis usually have enhanced staining reaction. These should be taken into account when assessing the Papanicolaou staining (see Fig 2).

Pale Hematoxylin staining will result not only in pale nuclei but will also cause patchy cytoplasmic staining (see Fig 3). Poor hematoxylin staining can be a cause of missed high grade

lesions (see Fig 4). Figure 5 is an example of poor nuclear staining in a cytolytic smear. Figure 6 is an example of good nuclear staining in a cytolytic smear with crisp and distinct nuclei. Overstaining in hematoxylin can result in poor nuclear to cytoplasmic differentiation and excessive background staining (see Fig 7).

Exhausted bluing agent can also cause poor nuclear staining resulting in reddish nuclei (see Fig 8). In the regressive Pap staining method, ethanol ammonia is used to convert the nuclei from red to blue/purple.

Poorly prepared or exhausted EA can result in dull cytoplasmic staining and lack of contrasting cytoplasmic colours (see Fig 9). A dull pink and degenerate appearance may occur with smears that accompany histology specimens. It is usually due to formalin fixation (see Fig 10).

TROUBLESHOOTING THE PAP STAIN

PROBLEM	POSSIBLE REASON	REMEDY
DARK NUCLEI	Too much time in Harris Hematoxylin. Not enough time in HCl or HCl concentration less than recommended.	Reduce time in HCl by 10,15,20,30 sec intervals. Increase time in acid by 5,10 sec.
PALE NUCLEI	Polyethylene glycol coating not removed from cells prior to Hematoxylin. Concentration of HCl greater than recommended or too much time in HCl. Not enough time in Hematoxylin. Hematoxylin diluted by water (if water not properly drained from slides). Stain not changed frequently enough resulting in Hematoxylin exhausted.	Extend pre-staining soak with aqueous ethanol. Reduce time in acid by 5,10 sec and ensure correct amount of acid is added to the solution. Increase time in HCl by 10,15,20,30 sec intervals. Ensure the arm of the staining machine is operating correctly. Ensure a set amount of slides are stained and then stains are changed.
CYTOPLASMIC COLOUR NOT CONSISTENT	Air drying prior to fixation. Polyethylene coating inadequately removed from cells. Solutions not at proper level within staining dishes. Excessive time in Hematoxylin or Hematoxylin not removed prior to OG and EA dyes. Slides left too long in ethanol rinses or clearing solutions following OG and EA. Inadequate rinsing of slides between solutions. Insufficient rinsing following staining solutions. pH of tap and distilled water not sufficiently alkaline. pH of EA needs to be controlled (pH 4.5 to 5 achieves maximum result). EA dye exhausted.	Report the findings to the referring clinician. Extend pre-staining soak in aqueous ethanol. Check staining solution level. Reduce time in HCl by 10,15,20,30 sec intervals. Reduce ethanol rinse time. Check if ethanol is changed regularly. Increase ethanol rinse time. Check pH. Check pH. Ensure a set amount of slides are stained and then stains are changed.
MACROSCOPICALLY ALL SLIDES ARE PINK, ORANGE OR YELLOW	Slide drying oven temperature too high.	If this happens there is nothing that can be done to obtain a well-stained sample.
DULL PINK AND DEGENERATE APPEARANCE	This usually occurs to smears that accompany histology specimens. It is usually due to formalin fixation.	Ensure formalin jet and smear is transported in separate bags.
DULL GREYISH APPEARANCE OF CELLS	Water contamination of dehydrating and clearing solutions. Polyethylene glycol coating not removed from cells prior to staining of filter.	Ensure dehydrating and clearing solutions are changed regularly. Extend the pre-staining soak time.
OPAQUE/WHITE COLOUR ON BACK OF SLIDE	Bluing agent not rinsed from slides.	Use two separate but thorough water rinses following Scott's Tap Water substitute. (For progressive Pap staining).
STAIN DEPOSIT	Staining dyes not changed or filtered properly.	Ensure staining dyes changed or filtered regularly.
FUNGAL CONTAMINATION	Slides contaminated by fungus during the staining process.	Change staining solutions regularly and ensure the staining containers are disinfected with a dilute bleach solution.

LABORATORY SAFETY

While getting optimal staining results is imperative, laboratory safety factors is an area not to be overlooked. The following must be considered:

- Wear appropriate protective clothing in the lab, a laboratory coat or gown is essential.
- Wear sensible shoes which protect the feet from spills.
- Wear gloves to protect hands. Nitrile gloves can be worn when handling xylene. Rubber gloves are easily worn out by xylene.
- Never smoke, eat or drink in a laboratory.
- Stain and coverslip in a well ventilated area preferably using fume extraction.
- Wash hands thoroughly after contact with chemicals, dyes or human or animal tissues.
- Store incompatible chemicals in separate areas of the laboratory.
- Have xylene levels monitored.
- Fume cupboards should be tested and serviced regularly.
- Clean up spills immediately.

CONCLUSION

Despite more than 50 years of widespread usage, problems can still be encountered with the Pap-stain. Staining results vary considerably between laboratories and sometimes within a laboratory from day to day. Good staining results are required for the detection and categorisation of cellular abnormalities. To achieve this, quality control of staining procedures needs to be established within a laboratory. Part of this is a comprehensive knowledge and understanding of the staining process. This will ensure that laboratory operators achieve optimal staining results. Something we are all dying to get right!!

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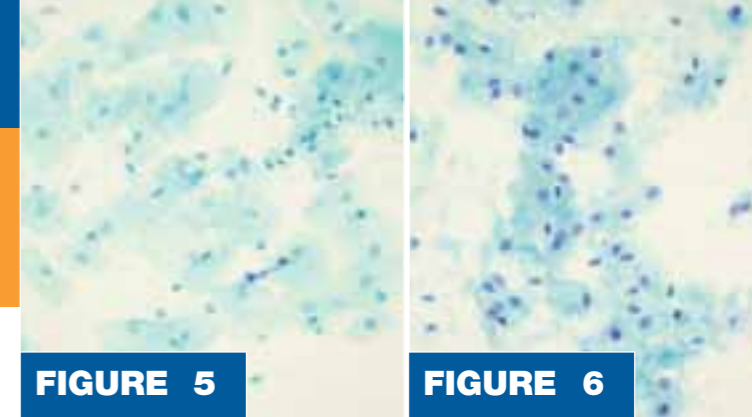


FIGURE 5

FIGURE 6

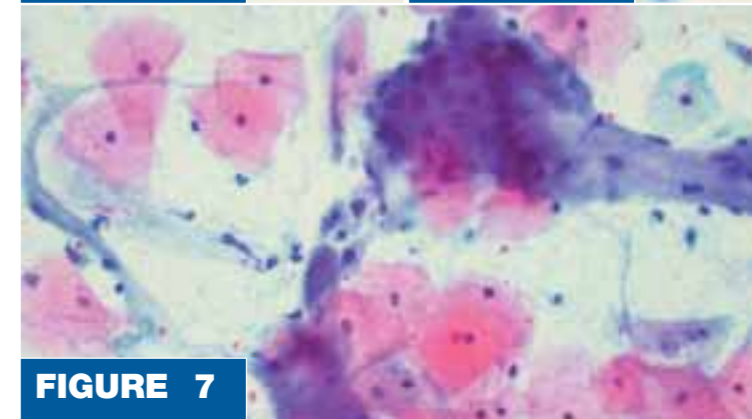


FIGURE 7

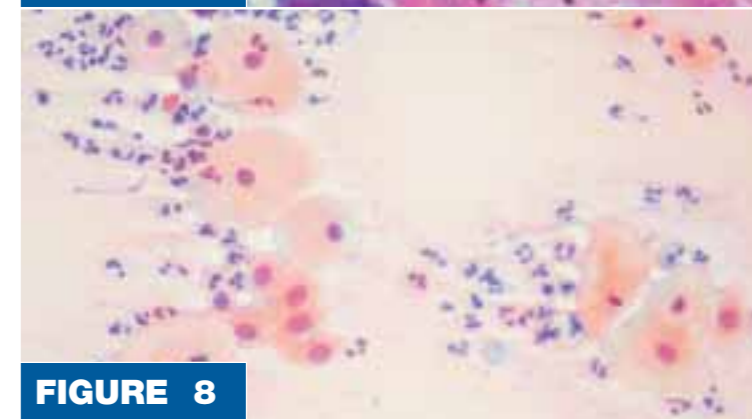


FIGURE 8

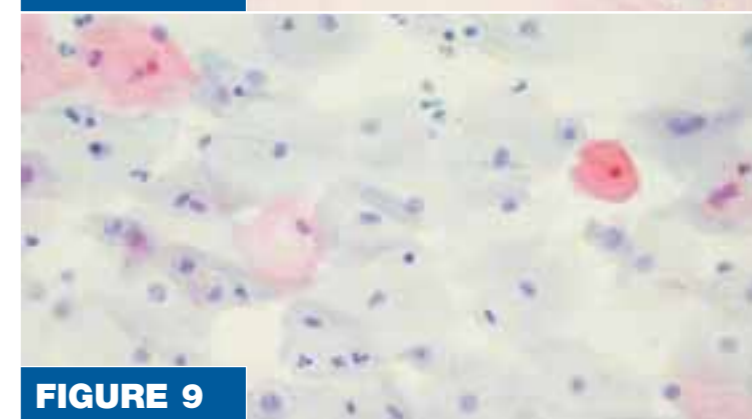


FIGURE 9

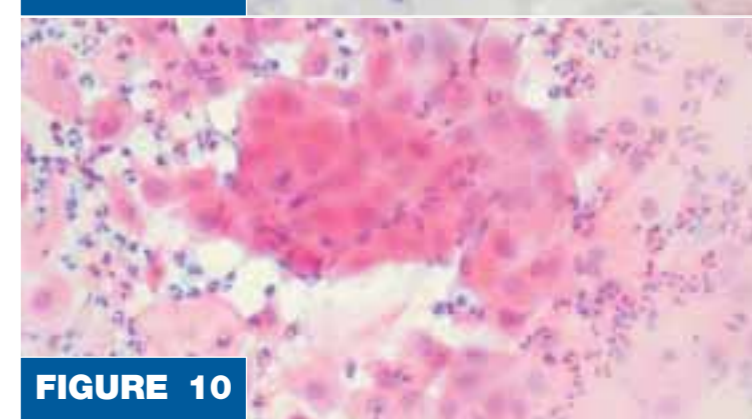


FIGURE 10