

A Quick Guide to Cytological Staining

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Cytology is the science of the interpretation of cells removed from the human body through clinical procedures or exfoliation. One of the most widely used groups of stains in cytology, the Papanicolaou series of stains, was developed by Dr. George N. Papanicolaou. These stains impart a characteristic color to nuclei and cytoplasmic components.

Some morphological changes and lesions are clinically easy to recognize, while other diseases or conditions produce cellular, molecular, or clinical signs that are difficult to identify. Cytological staining, which artificially colors cells, can aid researchers and clinicians in the screening, identification, and diagnosis of a number of pathological conditions, including infection, inflammatory diseases, and cancer.

Cytological assays provide researchers and clinicians with a relatively simple and fast diagnostic tool that is widely accepted in the scientific community. However, cytology assay methods must be consistent and reliable as cytologists rely heavily on the quality and appearance of a stain for interpretation.

Characteristics of Staining

Different stains react to, or concentrate in, different parts of a cell or tissue, and these unique staining properties can be used to reveal specific organelles or areas of interest. Additionally, combinations of stains are often used to reveal more detail than a single stain alone. The staining itself can be done either *in vivo*, in the case of studying the morphology or localization of whole organisms, live tissue, or cells; or *in vitro*, where cells or cellular components have been collected from their biological context.

A common combination of stains includes using a 'stain of interest' and a counterstain to enhance contrast and differentiate between cellular components. Two of the most commonly used cytological stains are:

- Papanicolaou (PAP) stain - Papanicolaou stain is a modified hematoxylin and eosin (H&E) stain and is recommended for the staining of alcohol fixed cytology slides¹.
- Romanowsky stains – Romanowsky stains exist in many variants and are primarily applied to air-dried smears but may also be used for wet fixed slides¹.

Both stains are popular in cytopathology laboratories for revealing structures such as the nucleus, cytoplasm, and cellular granules. However, staining air-dried cells with a Romanowsky stain allows for a greater size evaluation of the nucleus, cytoplasm and overall cell area².

Sample Preparation

Processing samples for cytological staining includes specimen collection, preparation of tissue/cellular slides for microscopic examination, staining, and screening. The actual preparatory steps involved depend on the particular application; some or all of the following procedures may be required and subjected to quality control and quality assurance measures.

- Smear – In a smear specimen, samples are obtained from the epithelial surface of organs, internal organs, or bodily fluids. Following specimen collection, fixation may be carried out in accordance to sample requirements and standard laboratory procedures. For example, blood or bone marrow samples can be prepared fresh or from sample collection tubes containing ethylenediaminetetraacetic acid (EDTA).

- Fixation – Chemical fixation of samples aims to preserve the morphology of the cells or tissue. Common chemical fixatives include formaldehyde, ethanol, and methanol. Fixation can be achieved by complete saturation or immersion of a specimen slide in an alcohol-based fixative for 15 to 20 minutes. A quick fixation can also be performed using a spray fixative that consists of alcohol and an extra carbowax protective coat over the slide, which is later removed before staining. Following fixation, whole tissue samples can be embedded in paraffin wax before microtome sectioning and slide preparation.
- Permeabilization – Permeabilization involves treating specimens with a mild surfactant to dissolve lipids from the cell membrane, thereby allowing stains to dye intracellular components and organelles. Antibodies used to detect intracellular antigens are often prepared in permeabilization buffer.
- Slide Mounting – Following staining, slides are cover-slipped with a ‘mountant’. The mountant usually comprises of a base constituent and an antifade agent. This permits the formation of a stable bond between the slide and coverslip, and also protects the sample from air drying or shrinkage and prevents oxidation or fading of the stain.

Papanicolaou (PAP) Staining

The universal stain for cytological samples is the PAP stain developed by the Greek cytopathologist, George Papanicolaou. He developed the polychrome staining reaction to study cell components, variations in cellular maturity, and metabolic activity. The PAP staining method comprises of a number of synthetic dyes that are either acidic (anionic) or basic (cationic). The basic dyes have an affinity for basophilic components with a net negative charge such as nuclei and ribosomes; whilst the acidic dyes have an affinity for acidophilic components with a net positive charge such as the cytoplasm, mitochondria and cilia¹.

PAP staining is a reliable technique and widely used in cytological staining and cervical cancer screening. The main advantages for PAP staining of cytological smears are:

- Well-characterized nuclei (Figure 1)
- Cytoplasmic translucency and identification of individual cells that are overlapping (Figure 1)
- Differentiation between acidophilic and basophilic cells

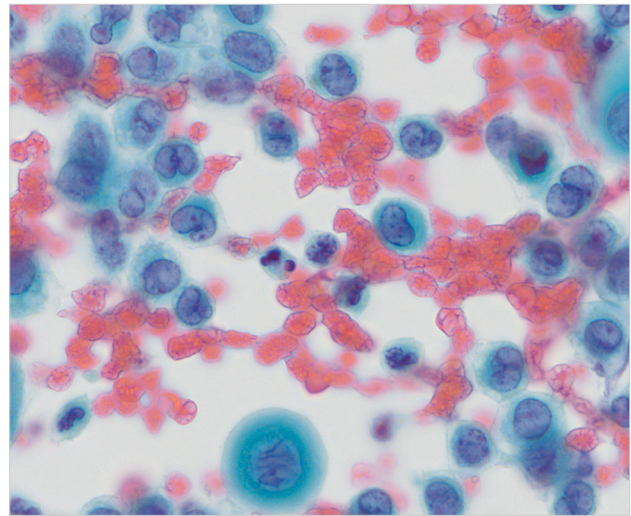


Figure 1 PAP staining revealing cellular samples

Common Dyes used in PAP Staining

The polychromatic PAP stain may include up to six dye preparations in three separate solutions (Table 1). The PAP staining method is a combination of a nuclear stain (hematoxylin) and two counterstains, Orange Green 6 (OG-6) and Eosin Azure 50 (EA-50; Figure 2)³. Hematoxylin stains nucleic acids and proteins and this hematoxylin dye component in a PAP stain can vary in both composition and content. OG-6 stains keratin, while EA-50 (a double stain – eosin and azure) stains the cytoplasm of squamous epithelial cells, nucleoli, and red blood cells. Both OG-6 and EA-50 have a high solvent concentration that provides cytoplasmic transparency and aids in the visualization of overlapping cells.

PAP staining may follow either progressive or regressive nuclear staining techniques, with the former being more commonly used protocol. In the progressive method, less concentrated hematoxylin is used to slowly stain the nucleus. The original red nuclear staining is then converted to a purple-blue color through immersion techniques by applying a bluing agent such as an alkaline solution. In the regressive staining method, non-acidified hematoxylin is used to over-stain the nucleus and then excess stain is removed by adding an acidic solution. In this method, a higher concentration of hematoxylin is used to perform a faster regressive stain and, in addition, removal of the excess stain provides greater background transparency. The de-staining is stopped by immersing slides in running tap water. The quality of de-staining may also affect the chromatic status in staining results³.

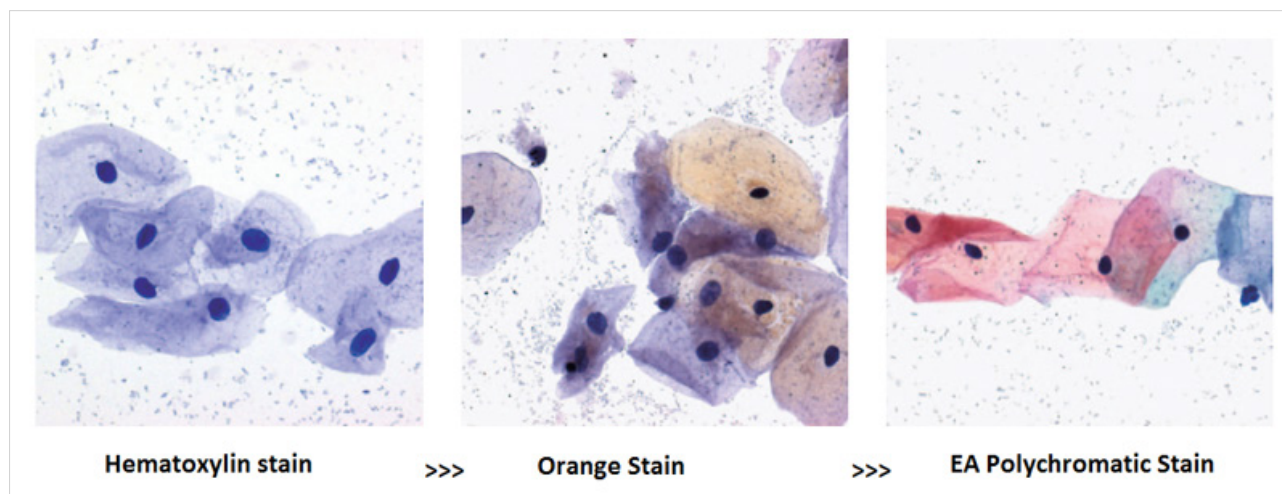


Figure 2 Polychromatic PAP stain

Hematoxylin is the optimum nuclear stain and the combination of OG-6 and EA-50 give the subtle range of green, blue, and pink hues to the cell cytoplasm (Figure 2). Bismarck brown Y is sometimes added to the EA-50 formulations. Although this has no effect on the staining pattern and color, it precipitates phosphotungstic acid which is responsible for the differential staining of OG-6 and EA-50⁴.

PAP-stained specimens will exhibit a range of colors from red to violet. The cell nuclei and chromatin patterns are typically blue to black on a well-prepared specimen. The PAP stain also aids in the determination of overlapping cells and distinguishing individual cells in thicker tissue sections. Keratin and glycogen enriched cells display a yellow hue; superficial cells give orange to pink hue; intermediate and parabasal cells stain turquoise green to blue hue; and metaplastic cells often show both green and pink hue.

Solutions	Formula	Principles
Gill's half-oxidized hematoxylin	Hematoxylin: 2 g Ethylene glycol: 250 ml Aluminum ammonium sulfate (alum): 17.6 g Distilled water: 730 ml Sodium iodate: 0.2 g Glacial acetic acid: 20 ml ⁴	Hematoxylin dyes attach to sulfate groups on the DNA. Hematoxylin stains cell nuclei as blue.
Orange Green 6 (OG-6)	Orange G (10% aqueous): 20 ml 95% ethanol: 980 ml Phosphotungstic acid: 0.15 g ⁴	First acidic counterstaining gives the cytoplasm orange color in matured and keratinized cells.
Eosin Azure 50 (EA-50)	Light green (3% aqueous): 10 ml Eosin Y (20% aqueous): 20 ml Phosphotungstic acid: 4 g 95% ethanol: 700 ml Methanol: 250 ml Glacial acetic acid: 20 ml ⁴	Second counterstaining is followed by EA-50 which is often a polychrome mixture of eosin Y and light green SF. Eosin Y stains the cytoplasm as pink in mature squamous cells, nucleoli, cilia, and red blood cells. Light green SF stains the cytoplasm as blue in metabolically active cells.

Table 1 Overview of commonly used dyes in PAP staining

PAP stains are commercially available from a number of manufacturers, but due to batch-to-batch variations in hematoxylin, many laboratories use commercially available stains with their own modifications. The method may also be modified to ensure optimal staining, as dye incubation times, variations in pH and temperature, and the number of slides stained can all affect the color of the stains. Maintaining staining consistency through quality control is considered a part of Good Laboratory Practice (GLP). Small degrees of color variation are acceptable provided that nuclear detail is well defined and the transparency of the cytoplasm is maintained.

PAP staining may be used in conjunction with the so-called 'special stains' (staining other than H&E) for further investigation and interpretation of cytological samples. For example, Giemsa stain can be used on blood smears and bone marrow samples to detect the presence of pathogenic bacteria; Prussian blue stain can detect iron in bone marrow and liver specimens; and Congo red stain detects amyloid deposits in bone marrow. Special stains may independently identify specific tissue characteristics in samples that could assist in an accurate cytological diagnosis.

The PAP smear test for identification of cervical cancer is one of the most widely used cancer screening methods. Following the introduction of George Papanicolaou's staining method in the 1950s, the incidence of cervical cancer and mortality in the United States has declined by more than 70%⁵. PAP staining continues to play an important role in the identification and diagnosis of a number of pathological conditions.

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