Nuances of the Papanicolaou stain

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THE PAPANICOLAOU (PAP) STAIN – STAIN PREPARATION AND PROCEDURE IN DETAILS

Among the major achievements in the history of cytopathology, the eponymous Pap stain method formalized by Dr. George N. Papanicolaou in 1942 was foundational. The Pap stain has been used all over the world since half a century for the staining of cervicovaginal smear. Many modifications have been published after George Papanicolaou described the original staining technique in 1942. The staining procedure varies with various staining protocols and methodologies used in different laboratories. The Pap stain is a polychromatic counterstaining method consisting of stains such as Orange G 6 (OG6) and modified eosin azure (EA). The strength of the Pap stain is such that it results in:

1. Well-stained nuclear chromatin
2. Differential polychromatic counterstaining of cytoplasm
3. Cytoplasmic transparency.

To achieve this, proper fixation of the smear is one of the most important prerequisite factors.

FIXATION AND FIXATIVES

The purpose of cytological fixatives is to maintain the cytomorphic characteristics and diagnostically essential elements of the cell. Ethyl alcohol is the fixative specifically recommended for cytological preparations. Fixation coarsens the cell structures and sharpens nuclear chromatin pattern and its details. Commonly used cytological fixatives include wet fixatives and dry fixatives.

WET FIXATIVES

1. 95% ethyl alcohol and ether in equal volume. Nowadays, it is not used as it is inflammable in nature and has a pungent odor
2. 95% ethanol: Fixation for routine cytological smear
3. 100% methanol
4. 95% denatured alcohol
5. 80% isopropanol
6. 80% propanol.

The cytology laboratory of G.M.C, Nagpur, uses 95% ethanol as the fixative for routine cytological smears for both cervicovaginal and fine-needle aspiration cytology smears. The slides should be fixed immediately in the fixative solution, as even a slightest air drying of the smear can alter cytomorphological features causing diagnostic problems. Prolonged fixation for several days or even weeks will not alter cellular features. Fixative solution along with the smear may be refrigerated in such situations to minimize the evaporation.

DRY OR COATING FIXATIVES

Dry or coating fixatives are major substitutes for wet fixatives in special situations like cancer detection camps or when transportation of smears from distant collection centers is required. These fixatives are available commercially and are either aerosol (applied by spraying) or liquid based (dropped over a smear). They are composed of an alcohol base, which fixes the cells and a wax-like substance that forms a thin protective coating over the smear. Any ordinary hair spray can be substituted as coating fixative.

Method

1. The Pap smear is prepared in the usual way and immediately fixed with “dry fix” spray; or a few drops of liquid based fixative solution are put on the smear
2. While applying the spray, the bottle must be held at least 10–12 inches away from glass slide, which will prevent layering and hole formation
3. The slide is then placed on a flat surface for a few minutes to allow the “dry fix” to dry
4. The coating fixatives must be removed from the smear before staining. For this, slides are kept in 80% ethyl alcohol for 1–2 h. Sometimes, two changes of 80%
alcohol may be required to remove the coating fixative completely. If the coating fixative is not removed entirely before staining, it gives a "bubbling" effect to the smear which interferes with the diagnosis.[2]

STAIN PREPARATION

Nuclear stain hematoxylin

Hematoxylin is the most widely used nuclear stain. It is commercially available as an amorphous brown-colored powder form, which is extracted from heartwood of Central America Logwood – “Haematoxylum campechianum.”

- Hematoxylin itself is not a dye, it has to be oxidized to "hematin" which is actual staining component
- The natural oxidation process or ripening of hematoxylin in aqueous solution takes place gradually over a period of time by keeping the stain bottle in the sunlight area at least for a month
- The instant ripening can be achieved chemically by the addition of oxidizing agents such as sodium iodate or mercuric oxide
- Hematoxylin stains the DNA and RNA of the cell
- This stain can be used either progressively (Box 1) (without the use of acid alcohol differentiation) or regressively (Box 2)
- In the bluing step (Box 3), the absorbance peak of hematoxylin is shifted visually resulting in the change of color from red to blue
- Harris, Gill's, and Mayer's reagents are the most commonly used alum hematoxylin (Box 4) in cytology laboratory.

The cytology laboratory of G.M.C.H, Nagpur, uses the regressive method of staining using Harris and Gill's hematoxylin followed by differentiation with 0.5% acid alcohol and bluing in the running tap water.

Box 1: Progressive staining

- Direct staining method
- Stain is applied to tissue in strict sequence for specific time
- Controlled by observation under microscope

Box 2: Regressive staining

- Indirect staining method
- Tissue is overstained
- Excess stain is selectively removed from all except the structures to be demonstrated.
- Excess stain removed by acid alcohol to achieve desired staining
- This process of selectively removal of excess stain is called Differentiation

Box 3: Bluing

- Alum hematoxylin stain red color to the nuclei, which is converted to blue black when section is washed in weak alkali solution like tap water/ Scott's tap water/ saturated lithium carbonate/ 0.05% ammonia in distilled water / etc.

Box 4: Alum Hematoxylin

- Alum is a chemical compound containing double sulfate salts of metals like aluminum (most common), iron or chromium.
- Common alum used in laboratories are: Ammonium aluminum sulfate (i.e., ammonium alum) and potassium sulfate (i.e., potassium alum).
- Alum is a Mordant (Box 5) used for hematoxylin.
- Different alum hematoxylin are Harris Hematoxylin, Mayer's Hematoxylin, Gill's Hematoxylin, Ehrlich's Hematoxylin, etc.

Box 5: Mordants

- The oxidation product of hematoxylin is Hematin.
- Hematin is a natural dye having color properties.
- However, this Hematin has poor affinity for tissues and does not stain nuclei when used alone.
- This affinity of Hematin to tissues is improved by use of Mordants.
- Mordant is a substance or a metal which chemically binds with the dye and acts as a link between dye and tissue.
- Most commonly used mordants for Hematoxylin are salts of Aluminum (Alum), Iron, Tungsten, Lead, etc.

Harris hematoxylin: (For 1 L)

- Hematoxylin powder 5 gm
  (Yucca diagnostics, MERCK)
- Absolute methanol/ethanol (solvent) 50 ml
- Mercuric oxide (oxidizing agent) 2.5 gm
- Aluminum ammonium sulfate (mordant) 100 gm
- Glacial acetic acid* 40 ml
- Distilled water 1000 ml

Note: *Glacial acetic acid is used as a stabilizer and slows down the oxidation process.

Preparation

1. Dissolve hematoxylin in alcohol
2. Dissolve alum in water and bring to boil
3. Add dissolved hematoxylin to alum solution and again bring to boil
4. Remove the flask from heat and immediately add mercuric oxide
5. Stir the solution until a dark purple color appears
6. Cool the flask in water bath
7. Filter and store in dark bottle in refrigerator.

Principle

- Being a regressive stain, Harris hematoxylin over stains the nucleus and the excess stain is removed with the help of running tap water followed by differentiation using 0.5% acid alcohol
- The decolorizing acid is then removed by keeping the slides in running tap water for bluing
- Timing in acid alcohol is essential for the final appearance of the nuclear chromatin
• If acid bath is inadequate, the contrast between the chromatin and the parachromatin is less and uptake of the counterstain is also lessened.

**Gill’s hematoxylin**

Gill’s hematoxylin is used for the procedure of PAP stain. For the preparation of 1 L of Gill’s hematoxylin, the following chemicals are combined in large conical flask in the given order. Stir the mixture for an hour manually or preferably on a magnetic stirrer at room temperature.

**Preparation**

1. Distilled water 730 ml  
2. Ethylene glycol 250 ml  
3. Hematoxylin 2 g  
4. Sodium iodate (oxidizing agent) 0.2 gm  
5. Aluminum sulfate (mordant) 17.6 gm  
6. Glacial acetic acid* 20 ml  

*Glacial acetic acid stabilizes aluminum-hematin complex and slows down the oxidation of dye.

- Gill’s hematoxylin can be prepared easily and rapidly
- The unoxidized hematoxylin forms a metallic scum on the surface of the stain, which can interfere with the staining and precipitate on the slides
- Although no surface or bottom precipitate is recovered, it is a good practice to filter the stain, before staining
- Fresh hematoxylin should be added when the level in staining jar drops
- Being a progressive stain, Gill’s hematoxylin stains the nucleus to the desired intensity and this is followed by bluing
- The optimum time for nuclear staining is 1–1½ min
- If Gill’s hematoxylin is used as a regressive stain, then the time of nuclear staining is increased up to 10–15 min followed by differentiation using acid alcohol (0.5 %) and then bluing in running tap water or in lithium carbonate bath.

**OG-6 modified (Gill’s modified OG-6)**

- It is a cytoplasmic counterstain
- In OG-6, OG signifies Orange G ("G" is an abbreviation for German word “gelb” which means yellow) and 6 denotes the concentration of phosphotungstic acid added
- OG is an acidic protein dye, which can be combined with other protein dyes
- Other variants of OG are OG-5 and OG-8
- Modified OG is a Gill’s modification and is a combination of OG and phosphotungstic acid. It minimizes precipitation and reduces filtering and staining time
- Gill’s modified OG is stable in solution and gives high-quality staining results.

**EA modified (Gill’s modified EA)**

- It is also a cytoplasmic counterstain
- EA-65 is a polychromatic stain and contains 3 dyes: Eosin Y, Light Green SF yellowish, and Bismarck Brown Y
- The number of light green and eosin molecules must be adjusted relatively to one another to ensure the balanced staining
- Eosin is purely an acidic dye and binds mainly to protein
- EA is followed by a number which denotes proportion of the dyes. There are different formulations of this product (EA36, EA50, and EA65). EA-65 contains higher percentage of Green stain component, also referred to as "PAP EA65 enhanced green."
- In EA modified formula, Bismarck brown has been eliminated.

Although not numbered, Gill’s modified EA is similar to EA-36 and EA-50, but offers improved staining performance.

The main ingredients are

1. Light Green* 2 g  
2. Eosin** (LOBA) 2 g  
3. Phosphotungstic acid*** 1 g  
4. Distill water 480 ml  
5. 95% Ethanol 500 ml  
6. Glacial acetic acid**** 20 ml  

Dissolve light green, eosin, and phosphotungstic acid in distilled water separately. Then, mix all the three solutions together and add 95% ethanol and glacial acetic acid. Mix them thoroughly.
· EA modified contains light green and eosin only
· In EA modified formula, Bismarck brown has been deleted as it is considered to have no distinguishable color effect. Combination of Bismarck brown and phosphotungstic acid create precipitation, alters color over time and reduces shelf life

*Light green stains metabolically active cells, that is, parabasal and intermediate cells, histiocytes, columnar cells, and malignant cells. **Eosin stains the mature squamous cells, erythrocytes, nuclei, and cilia. Red cells stain blue green if eosin is exhausted. ***Phosphotungstic acid is added to adjust the pH of the stains and helps optimize the color intensity. It selectively excludes eosin from cytoplasm of certain cell types and permits it to be stained by Light Green or Fast Green. The sites remaining unstained by hematoxylin, OG, and Light Green are then stained by eosin.

****Glacial acetic acid is included in formula as it gives better cytoplasmic color differentiation and less background staining. It maintains required pH for optimal staining.

Acid alcohol
0.5% acid alcohol.

Preparation
1. Conc. HCL 5 ml
2. 70% ethanol or rectified spirit 995 ml.

Bluing agent
Scott’s tap water substitute (STWS), dilute aqueous solution of ammonium hydroxide, and lithium carbonate are the most commonly used bluing agents. Tap water may serve as a bluing agent if the water pH is higher than 8.

STWS - pH 8.02
1. Distilled water 1000 ml
2. Magnesium sulfate MgSO4 10 gm
3. Sodium bicarbonate NaHCO3 2 gm.
(20 g of MgSO47H2O (Epsom salt) can be used instead of 10 g of magnesium sulfate). Mix the ingredients in water and stir them well. STWS should be discarded after each round of staining.

Other bluing agents
1. 3 ml liquid ammonia is added to 97 ml of 70% ethanol 
2. Dissolve 1.5 g of lithium carbonate in 100 ml of distilled water (stock solution) and add 30 drops of this solution to 1000 ml of distilled water.

Stain maintenance
Stain maintenance is necessary to remove contaminants, floaters and to restore the activity of the stain.

· Filter the stain daily: It is recommended because washed off cells may be a source of contamination. Hematoxylin forms a scum and OG may precipitate into crystals
· Change the staining solutions:
  a. Hematoxylin: It has a long shelf life. Replace with fresh stain after 1500 slides are stained
  b. OG modified: Replace after 2000 slides have been stained. This too has a long shelf life
  c. EA modified: It has a short shelf life and so replace it after 1500 slides have been stained
  d. Xylene: Filter through filter paper to remove contaminant cells
  e. Alcohol: One jar should be replaced daily.

Staining procedure: [Manual method followed in GMC, Nagpur. Figure 1]
From the fixative, the slides are passed through the following solutions

| Title                          | Time     |
|-------------------------------|----------|
| Running tap water             | 2–3 min  |
| Gills hematoxylin             | 10–15 min|
| Running tap water             | 2–3 min  |
| 0.5% acid alcohol             | 1 Dip    |
| Running tap water             | 3–5 min  |
| 95% ethanol                   | 10 Dips  |
| Orange G modified             | 2–3 Dips |
| 95% ethanol                   | 2–3 Dips |
| EA modified                   | 2–3 Dips |
| Absolute alcohol              | 10 Dips  |
| Dry the slides                |          |
| Xylene                        | 3–5 min  |
| EA: Eosin azure                |          |

Mount the slides in dibutylphthalate polystyrene xylene (DPX).

Automated stainer (Method followed in GMC, Nagpur)
From the fixative, the slides are taken out and fixed in the slide carrier of the automatic robotic arm of the automatic stainer. The following program is followed.

| Running tap water             | 1 min    |
| Gills hematoxylin             | 15 min   |
| Running tap water             | 5 min    |
| 0.5% acid alcohol             | 1 Dip    |
| Running tap water             | 5 min    |
| 95% ethanol                   | 1 min    |
| Orange G modified             | 1 Dip    |
Results

1. Nuclear chromatin is stained as classical blue to purple in color
2. RNA associated with DNA is delicately stained so that nucleoli display their characteristic eosinophilia
3. Bar bodies are stained conspicuously
4. Mucus and cell debris are minimally stained, thereby exhibiting an unobscuring background
5. The first counterstain, OG-6 modified stains keratin a brilliant orange, yellow, or brown
6. The second counter-stain EA-36stains the cytoplasm of superficial cells (cornified) various shades of pink
7. Cytoplasm of the intermediate cells (non-cornified) stains pale blue or green
8. Cytoplasm of parabasal cells stains deep green
9. Candida stains red
10. Trichomonas vaginalis stains grey green.

Important factors for optimum staining

Excellence of the PAP’s staining depends largely on the delicate tints and transparency of the stained cells. The quality of the stained slides is dependent on the staining time, solubility and percentage of the dye concentration that is used in making nuclear and cytoplasmic stains. The following factors are useful in maintaining this desired quality.

1. The smears should not be allowed to dry at any time/ between any steps
2. Prolonged standings of the stained smears in the final grades of alcohols should be avoided as it leads to destaining of the cytoplasm
3. The stains should be kept well covered as water absorption causes a dense opaque stain. Evaporation of the alcohol changes the color balance and causes precipitation of the stain
4. Daily filtration of alcohol through a tight pad of cotton wool is recommended. This removes “free floating” cells or “floaters” thus minimizing cross-contamination
5. After staining approximately 800 slides, alcohol should be filtered with Whatman filter paper 1. This can be done at the weekends
6. Hematoxylin remains relatively constant in staining. Fresh stains should be added daily to replace stain loss due to evaporation
7. Daily filtration of xylene should be done through Whatman filter paper 1. This removes the moisture and floaters
8. The absolute alcohol and xylene of the last few steps must be fresh. All alcohol and xylene should be replaced by fresh if the following is noted
   a. A pale murky staining color
   b. Loss of sharp contrast and staining reaction of nuclear chromatin and cytoplasm
   c. Microscopic water droplets on the smear
9. When manual staining is done, it is a good practice to blot the slides carrier on a thick pile of blotting paper for a few seconds. This will minimize transfer of stain and alcohol from one jar to another and also save the solution
10. Shelf life of the stains may be increased by storing them in dark (amber) colored bottles when not in use and keeping the staining jar covered
11. Purchasing readymade stain is time saving exercise but they are not cost effective. Furthermore, the composition and shelf life are unknown and hence the staining results are not optimal.

DESTAINING AND RESTAINING PROCEDURE

Occasionally, it is desirable to destain and restain poorly stained slides or when special stains are required. They can be destained and restained after the removal of coverslip as follows.[2]

1. The coverslips are removed by soaking the slides in xylene till the coverslip falls off
2. The slides are transferred into a jar containing absolute alcohol for a sufficiently long time (½ of h)
3. The slides are then placed in 1% acid alcohol solution till the smear is completely colorless. It may require a few dips to 1 min or even longer, depending on the thickness of the smear
4. The slides are gently and thoroughly washed in running tap water so that all the traces of acid is removed
5. Then the slides are stained by routine PAP staining technique or any other desired stain.

REHYDRATION TECHNIQUE FOR AIR-DRIED SMEARS

The rehydration procedure may be used for air dried smears stored upto 72 hours before staining with HE and Pap.[6] It must, however, be noted that squamous cells may be restored to a considerable extent after rehydration. Whereas cells of the secretory type often suffer irreparable damage. The simplest technique of rehydration is as follows:
1. Place the air-dried smears in 50% aqueous solution of glycerol for 3 min
2. Rinse the slides in two changes of 95% alcohol and then stain the slides by routine PAP stain.[1]

METHOD OF COVERSLEPPING

1. Place coverslips on clean blotting paper
2. Remove the smear from xylol, drain off the excess xylol
3. Place one or two drops of mounting medium DPX on the smear, invert the coverslip on the smear
4. Apply gentle pressure, so that the mounting medium will spread uniformly and to remove air bubbles squeeze out excess mounting medium
5. Using xylene moistened soft absorbent paper, wipe the edges of the slide clean
6. Lay the slide flat till dry.

COVERSLIP REMOVAL METHODS

1. Xylene at room temperature: Most commonly used solvent for coverslip removal. It dissolves DPX. Its turnaround time is more and takes 72–94 h for coverslip removal
2. Xylene at 56 degree Celsius: Slides are kept in glass jar containing xylene and immersed inside water bath maintained at 56°C.[3] Heat fastens the melting of DPX
3. Freezing: Fastest method of coverslip removal. Slides are kept in freezing chamber of domestic refrigerator at 0–4°C with coverslip facing downwards
4. Petrol: Dissolves DPX. Plastic airtight jar with cap is used
5. Diesel: Dissolves DPX. Plastic airtight jar with cap is used
6. Liquid nitrogen: Not easily available at the setup
7. Ultrasonic vibrations: May cause damage to the tissue section
8. Scratching coverslip along with application of ice block: Causes damage to smear/tissue section on the slide.

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None.

LIST OF ABBREVIATIONS (In alphabetic order)

DNA - Deoxyribonucleic acid
DPX - dibutylphthalate polystyrenexylene
EA - Eosin Azure
GMC - Government Medical College, Nagpur
HIMEDIA - company name
LOBA - company name
MERCK - company name
OG6 - Orange G 6
Pap - Papanicolaou
RNA - Ribonucleic acid
STWS - Scott’s tap water substitute

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