Протоколы окраски по Папаниколау I.

Подготовка к публикации, перевод и комментарии А.В. Безрукова.

1. Первый вариант протокола окраски по Г. Папаниколау (1942-1943 г.г.) [1,2].

A NEW PROCEDURE FOR STAINING VAGINAL SMEARS[1]

BY GEORGE N. PAPANICOLAOU

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In the course of a study of vaginal smears conducted in cooperation with Dr. Herbert F. Traut, of the Department of Gynecology of Cornell Medical College and of New York Hospital, for the purpose of diagnosing malignant tumors and other pathological conditions of the female genital tract,[2] it was realized that certain modifications and improvements in our procedure of staining vaginal smears found in menstrual and other uterine bleedings and have a pathognomonic value in the diagnosis of adenocarcinomas of the fundus[3] and of other gynecological conditions. These cells also have great importance in the evaluation of the normal menstrual cycle, of sterility, and of estrogenic and other endocrine therapy.

After long experimentation it was found that a much greater transparency and an equally good color differentiation of the cells can be obtained by the use of solutions of stains in 95 per cent alcohol instead of aqueous solutions. Various alcoholic stains were thus developed, but here only two will be described which are now being used more generally in our laboratory (see Table I).

TABLE I

			Stain EA 36	Stain EA 25
Light green SF yellowish	National Aniline and Chemical Co.	0.5 per cent solution in 95 per cent alcohol	45 cc.	44 cc.
Bismarck Brown	National Aniline and Chemical Co.	0.5 per cent solution in 95 per cent alcohol	10 cc.	12 cc.
Eosin yellowish	National Aniline and Chemical Co.	0.5 per cent solution in 95 per cent alcohol	45 cc.	44 cc.
Acid phosphotungstic	Merck		0.200 gm.	0.170 gm.
Lithium carbonate, saturated aqueous solution			1 drop	1 drop

were necessary. Methods which proved to be successful in other applications[2, 4, 5] were not found to be entirely satisfactory in this particular work because of a common disadvantage. The staining of the cells was too deep to permit a sharp definition of their outlines in smears that were relatively thick or contained much blood. In most cases of carcinomas and in many other pathological conditions there is a profuse vaginal discharge frequently mixed with blood which forms a heavy film on the slides. In such rich and bloody smears there is considerable crowding and overlapping of cells which, when deeply stained, can not be well differentiated. This applies more particularly to the small endometrial cells which are often

The 0.5 per cent alcoholic solutions are first prepared. As the solubility of the stains in 95 per cent alcohol is low, the solutions are heated at the time of preparation. The solutions are kept in stock without being filtered. Stains EA 36 or EA 25 should, however, be filtered in order to eliminate undissolved particles of stain.

[1] From the Department of Anatomy, Cornell University Medical College, New York, N.Y. Aided by a grant by the Commonwealth Fund.

[2] G. N. Papanicolaou and H. F. Traut, *Jour. Obst. and Gyn.*, 42:193, 1941.

[3] G. N. Papanicolaou, *Amer. Jour. Anat.*, 52:519, 1933.

[4] E. Shorr, *Science*, 91:321, 1940; *ibid.*, 91:579, 1940; *ibid.*, 94:545, 1941.

[5] G. N. Papanicolaou, *Jour. Lab. and Clin. Med.*, 26: 1200, 1941.

Рис.1. Копия страницы 1 репринта статьи [1] (между страницами 6 и 7 книги [2])

The staining procedure is as follows:

1. Fix smears immediately (before drying) in equal parts of 95 per cent alcohol and ether for 5 to 15 minutes.[6] Rinse in 70 per cent and 50 per cent alcohols and in distilled water.

2. Stain in hematoxylin for 5 to 10 minutes.[7] Rinse in distilled water. Rinse 3 to 4 times in 0.5 per cent aqueous solution of hydrochloric acid. Rinse thoroughly in water. Leave for one minute in a weak solution of lithium carbonate (3 drops of a saturated aqueous solution per 100 cc. of water). Rinse thoroughly in water.

3. Rinse in distilled water, then in 50 per cent, 70 per cent, 80 per cent, and 95 per cent alcohols.

4. Stain for one minute in the solution (OG 6) given below.[8]

5. Rinse 5 to 10 times in each of two jars containing 95 per cent alcohol, to remove excess stain.

6. Stain in EA 36 or EA 25 for 2 minutes.

7. Rinse 5 to 10 times in each of three jars containing 95 per cent alcohol. (Do not use the same alcohol which was used after Orange G.) Rinse in absolute alcohol and xylol. Mount in Clarite, Canada Balsam, or Gum Damar.

The advantages offered by this staining method are the following: (1) The epithelial cells and the erythrocytes are more transparent. Overlapping cells can be more easily differentiated. (2) The color of the acidophilic cells varies from red to orange. This helps in the identification of certain smear types. Basophilic cells stain green or blue-green. (3) Cells or fragments of tissue penetrated by blood take a characteristic orange or orange-green color which permits an easier recognition of small amounts of blood, even when erythrocytes are not distinctly seen. (4) Smears which were subjected to partial or even complete drying can be stained fairly satisfactorily. The differential coloring is not entirely lost.

Stains EA 36 or EA 25 can be used for short staining by those who want to make an immediate examination of a slide. No fixative needs to be used. The slides are dipped directly in the staining solution or covered by stain contained in a dropping-bottle. The smears are thus fixed and stained simultaneously within a few minutes, although they may be kept in the stain for a longer time without being overstained. The excess stain is washed off in 95 per cent alcohol and then the slides are carried through absolute alcohol and xylol and mounted in Clarite. The nuclei are stained faintly, but the cells show good differential staining and retain their transparency. Smears stained by this simple method can be restained by a repetition of the procedure described in this paper, including hematoxylin. This will improve the nuclear staining as well as the cellular differentiation and will permit the use of the same smears for a more detailed cytological study.

[6] Although smears may be kept in the fixative indefinitely, a prolonged fixation of a week or more affects the staining reaction of the cells.

[7] Staining for only 2 minutes is often sufficient, but, as a rule, better results are obtained with longer staining of 5 to 6 minutes for normal smears and of 8 to 10 for smears used for diagnostic purposes, more particularly for cancer diagnosis. For sections, even longer staining is advised. This timing applies more specifically to Harris Hematoxylin prepared with domestic hematoxylin and ammonium alum, which is now used in our laboratory. In order to obtain more uniform staining, used hematoxylin should not be discarded, but filtered from time to time. The loss from filtering and evaporation is gradually replaced by the addition of fresh stain.

[8] The addition of phosphotungstic acid to the Orange G solution intensifies the orange color. For normal slides a slight acidification of 0.010 gm. per 100 cc. (OG 8) or 0.015 gm. per 100 cc. (OG 6) is suggested. For cancer diagnosis a higher acidification of 0.025 gm. per 100 cc. (OG 5) is often preferable, as it gives a sharper contrast of the abnormal cell types.

Orange G	National Aniline and Chemical Co.	0.5 per cent solution in 95 per cent alcohol	100 cc.
Acid phosphotungstic	Merck		0.015 gm.

Рис.2. Копия страницы 2 репринта статьи [1] (между страницами 6 и 7 книги [2])

2. «Меморандум по окраске» - вариант методики, опубликованный в [3]

MEMORANDUM ON STAINING

Since the publication of the first part of the ATLAS in 1954, the OG-6-EA method used in our laboratory for staining smear preparations has remained essentially the same, except for a few modifications related chiefly to the preparation of the stains and the timing of the staining procedures. A description of our current method of staining is given here to provide readers with up-to-date information.

I. PREPARATION OF STAINS

1. Harris hematoxylin

Hematoxylin	5 gm.
Ammonium aluminum sulphate	100 gm.
Ethyl alcohol	50 cc.
Distilled water	1000 cc.
Mercuric oxide	2.5 gm.

Dissolve hematoxylin in alcohol. Add ammonium aluminum sulphate to water in a Pyrex beaker and heat to boiling point. Add hematoxylin solution and bring to a full boil once more. Remove from flame and add mercuric oxide at once. Swirl quickly until a black-purple color appears, a matter of seconds. Plunge beaker into cold water to cool rapidly. When cold, filter into a dark bottle. This is a stock Harris hematoxylin, to be aged not less than two weeks before using.

To prepare hematoxylin for staining add 4 cc. of glacial acetic acid per 100 cc. of stock Harris hematoxylin.

2. OG-6

First prepare a 10% aqueous stock solution of Orange G, using distilled water.

The OG-6 stain is prepared as follows:

95% Ethyl alcohol	950 cc.
Orange G - 10% stock solution	50 cc.
Phosphotungstic acid	0.15 gm.

3. EA-65

First prepare 10% aqueous stock solutions of: Light Green S.F. yellowish, Bismark Brown and Eosin yellowish (water and alcohol soluble).

Then prepare three alcoholic stock solutions as follows:

A. Light Green S.F. yellowish	0.05% solution in 95% alcohol
B. Bismark Brown	0.5% solution in 95% alcohol
C. Eosin yellowish	0.5% solution in 95% alcohol

The EA-65 stain is prepared as follows:

Alcoholic stock solution A	180 cc.
Alcoholic stock solution B	40 cc.
Alcoholic stock solution C	180 cc.
Phosphotungstic acid	2.4 gm.

Filter and store in brown bottle.

Note: All dyes used in these preparations are National Aniline and Chemical Company certified stains.

Рис.3. Копия первой страницы вставки (между страницами 12 и 13) книги [3]

II. STAINING PROCEDURE

1. After fixation, transfer slides, without drying, directly into 80% alcohol and run down through 70% and 50% alcohols to distilled water.
2. Stain in Harris hematoxylin for exactly 3/4 minute.
3. Rinse 3 times in distilled water, using 3 separate containers. All rinsing should be very gentle to prevent smears from being washed off slides.
4. Rinse in 50% alcohol.
5. Place in a solution of 1.5% ammonium hydroxide in 70% alcohol for one minute.
6. Rinse in 70% alcohol and run through 80% and 95% alcohols.
7. Stain in OG-6 for 1 1/4 minutes.
8. Rinse in 3 jars of 95% alcohol.
9. Stain in EA-65 for 3 minutes.
10. Rinse 3 times in 95% alcohol, using 3 separate containers.
11. Dehydrate and clear by running through:
    Absolute alcohol
    A mixture of equal parts of absolute alcohol and xylol
    Xylol

    Note: Fast clearing may be achieved by lifting the slide carrier out of xylol and allowing it to dry for a fraction of a second before putting it back in xylol.
12. Mount with Permount or other suitable mounting medium.

Our reasons for preferring this staining procedure are:

1. The total staining time is reduced to 12-15 minutes.
2. The relatively light staining of the cytoplasm permits a good transparency of the smears without impairing either the color differentiation of the various cell types or the sharp definition of the structural details of the nuclei.
3. Equally satisfactory results are obtained in all types of smears.

A technique for protecting smears from drying during shipping is described on pages 5 and 6 of the ATLAS. This method consists in dipping the freshly prepared smear into, or covering it with, a solution of 2 parts Diaphane to 3 parts of alcohol-ether. Its routine use in our laboratory during the past three years has shown it to be a very satisfactory and practicable procedure for all types of smears. A detailed description of this simple technique, which provides both a protective film for and a good fixation of the cells, can be found in the Journal of the American Medical Association, July 20, 1957.

G. N. Papanicolaou, M. D.

Рис.4. Копия второй страницы вставки (между страницами 12 и 13) книги [3]

3. Перевод (приведён перевод только протоколов окраски).

Таблица 1

1942-1943	1960-1963
Процедура окраски заключается в следующем: 1. Фиксируйте мазки немедленно (до их высыхания) в равных частях 95 % алкоголя и эфира в течение от 5 до 15 минут. Ополосните в 70 % и в 50 % спиртах и в дистиллированной воде. 2. Окрашивайте в гематоксилине от 5 до 10 минут. Ополосните в дистиллированной воде. Ополосните от 3 до 4 раз в 0,5 % водном растворе соляной кислоты. Тщательно промойте в воде. Оставьте на минуту в слабом растворе карбоната лития (3 капли насыщенного водного раствора на 100 см3 воды). Тщательно промойте в воде. 3. Ополосните в дистиллированной воде, затем в 50%, 70%, 80% и 95% спирте. 4. Окрашивайте до 1 минуты в растворе (OG 6), приведенном ниже. 5. Ополосните от 5 до 10 раз в каждом из двух сосудов, содержащих 95% спирт, для удаления избыточной краски. 6. Окрашивайте в EA 36 или в EA 25 до 2 минут. 7. Ополосните от 5 до 10 раз в каждом из трёх сосудов, содержащих 95% спирт. (Не используйте тот же спирт, который был использован после Оранжевого G). Ополосните в абсолютном спирте и ксилоле. Заключите в Clarite, Канадский бальзам, или Дамарлак.	II. Процедура окраски 1. После фиксации переместите слайды, не допуская высыхания, прямо в 80%-ный спирт и проведите через 70% и 50% спирты в дистиллированную воду. 2. Окрасьте гематоксилином по Гаррису точно в течение 3/4 минуты. 3. Промойте 3 раза в дистиллированной воде с использованием 3 отдельных ванн. Все ополаскивания должны быть очень нежными, для предотвращения смывания мазка со слайда. 4. Ополосните в 50%-ном спирте. 5. Поместите в раствор 1,5% гидроксида аммония в 70% спирте на одну минуту. 6. Ополосните в 70% спирте и проведите через 80% и 95% спирты. 7. Окрасьте в OG-6 в течение одной минуты с четвертью. 8. Промойте в 3 ваннах с 95% спиртом. 9. Окрасьте в EA-65 течение 3 минут. 10. Промойте 3 раза в 95%-ном спирте, используя 3 отдельных ванны. 11. Дегидратируйте и просветлите препарат, проведя его через: абсолютный спирт, смесь из равных

	частей абсолютного спирта и ксилола, ксилол. Примечание: быстрое просветление может быть достигнуто путем вынимания штатива со стёклами из ксилола на доли секунды для стекания жидкости и повторного опускания в ксилол. 12. Заключите препарат при помощи Permount или другой подходящей монтажной среды.

4. Комментарии

1. Методика впервые опубликована в статье Георгиса Папаниколау [1]. Пропись повторена в 1943 году в монографии [2]. В приведенном выше протоколе применена регрессивная окраска в гематоксилине, причём Г. Папаниколау не указывает, какой гематоксилин используется.

2. Вариант методики «Memorandum on staining» (Меморандум по окрашиванию) опубликован на вкладке между 12 и 13 страницами в наиболее значимой монографии Папаниколау «Атлас эксфолиативной цитологии» [3]. В приведенном протоколе применена прогрессивная окраска в гематоксилине, действительно, Г. Папаниколау указывает, что используется гематоксилин по Гаррису с добавкой уксусной кислоты, время выдержки в гематоксилине – всего три четверти минуты, дифференцирование в кислоте не применяется.

3. Преимуществом окраски Г. Папаниколау считает прозрачность мазка, при сравнительно слабой окраске цитоплазмы, нет ущерба дифференциации различных клеток по цвету цитоплазмы и разрешению тонкой структуры ядер. Согласно [7] выравнивание окраски цитоплазмы (а соответственно, и возможность «дифференциации различных клеток по цвету») происходит за довольно длительное время – 6-10 мин, что значительно больше, чем в прописях Г. Папаниколау. Таким образом, Г. Папаниколау выбрал компромиссный вариант методики с высокой прозрачностью, но с недостаточным окрашиванием и выравниванием окраски цитоплазмы клеток.

4. В примечании (низ правого столбца Таблицы 1), Г. Папаниколау предлагает для ускорения процесса применить приём окунание, широко использующийся в методиках в настоящее время.

5. До использования приведенных здесь методик Папаниколау использовал окраску гематоксилин – эозин с докраской красителем Водный синий и окраску по Шорру [4-6] (модификация трихрома по Массону), с которым работал в Корнельском университете. Эти методики и явились основой для разработки окраски по Папаниколау.

6. В рецептуре красок, приведенных Г. Папаниколау, согласно [7], присутствуют ошибки:

- слишком большое содержание красителя в OG 6 приводит к выпадению осадка;

- в красках ЕА краситель Бисмарк Браун реагирует с фосфорно-вольфрамовой кислотой, что так же приводит к выпадению осадка. В большинстве современных красок рецептура скорректирована.

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