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PERMANENT FEULGEN STAINING PREPARATION WITHOUT MOUNTING MEDIUM USE

Abstract. Introduction. *Feulgen staining is used in medical and biological studies when it is necessary to assess the level of DNA and its localization in cells. Some fixatives, mounting media and light can decrease the quality of preparations for microscopy by action on dye.*

Aim of the study. *Our experiment aimed i) to develop the Feulgen procedure modification that excluded the use of harsh fixation agents and mounting media and ii) to evaluate visually the effect of periodical light exposure during teaching process within 6 years onto Feulgen stained preparations quality.*

Materials and methods. *To escape the problem of stained specimens deterioration, we omitted the use of strong fixatives and mounting media, and avoided long light expositions on microscopic preparations. For permanent microscopic preparations production, the stained specimen covered with coverslip was properly air dried and then sealed by scotch tape on coverslip perimeter instead of mounting medium use. Preparations were kept wrapped in black paper and used for teaching of medical and biological students for eproximately 20 hours each year within a 6-years period. The microphotographs of the same site in tissue made at the start and end of this period were compared in quality visually.*

Results and discussion. *Air-drying of stained specimens between the glass slide and coverslip without mounting medium followed by their sealing with scotch tape provided preparations of good quality. Their use for teaching of students within 6 years during eproximately 20 hours per year caused no fading or other visually detectable changes in stained tissue. This was proved from comparison of microphotographs made at the start and end of this period.*

Conclusion. *Suggested variation of the Feulgen staining method can be applied for teaching of students in medicine and biology and, possibly, for routine analyses.*

Key words: *Feulgen staining, permanent preparations, no mounting medium.*

ПОСТІЙНИЙ ПРЕПАРАТ ФАРБУВАННЯ ЗА FEULGEN БЕЗ ВИКОРИСТАННЯ МОНТАЖНОГО СЕРЕДОВИЩА

Анотація. Вступ. *Фарбування за Фельгеном використовується в медичних і біологічних дослідженнях, коли необхідно оцінити рівень ДНК і її локалізацію в клітинах. Деякі фіксатори, монтажні середовища та світло можуть знизити якість препаратів для мікроскопії, впливаючи на барвник.*

Мета дослідження. *Наше дослідження мало на меті і) розробити модифікацію процедури Feulgen, яка виключала б використання жорстких фіксуючих засобів і монтажних середовищ та іі) оцінити візуально ефект на якість препаратів, забарвлених за Feulgen, періодичного освітлення під час навчального процесу протягом 6 років.*

Матеріали та методи. *Щоб уникнути проблеми погіршення пофарбованих зразків, ми не використовували сильні фіксатори та монтажні середовища, а також уникали тривалої експозиції світла на мікроскопічні препарати. Для ви-*

готовлення постійних мікроскопічних препаратів, пофарбований зразок, покритий накривним склом, добре висушували на повітрі, а потім заклеювали скотчем по периметру покривного скла замість використання монтажного середовища. Препарати зберігалися загорнутими в чорний папір і використовувалися для навчання студентів медичного та біологічного факультетів протягом близько 20 годин щороку протягом 6 років. Мікрофотографії однієї і тієї ж ділянки тканини, зроблені на початку та в кінці цього періоду, порівнювали за якістю візуально.

Результати і обговорення. *Висушування пофарбованих зразків без монтажного середовища між покривним і предметним скельцями на повітрі з наступним їх заклеюванням скотчем забезпечувало якісні препарати. Їх використання для навчання студентів протягом 6 років близько 20 годин щороку не викликало візуально помітних погіршень через вицвітання або інші зміни пофарбованої тканини. Про це свідчить порівняння мікрофотографій, зроблених на початку та в кінці цього періоду.*

Висновок. *Запропонований варіант методу забарвлення за Feulgen може бути застосований для навчання студентів медицині та біології та, можливо, для рутинних аналізів.*

Ключові слова: *Фарбування за Feulgen, постійні препарати, без монтажного середовища.*

Introduction. Around 100 years ago (during 1914–1924) Joachim Wilhelm Robert Feulgen, the German physician and chemist, invented the procedure for staining of DNA and DNA-containing materials, particularly, in microscopic preparations [5, p. 203] that is now called after him as Feulgen method. He also discovered congeniality of "thymonucleic acid" (now we name it as "DNA") of animals and plants [10, p. 46] and estimated the nature of nucleic acids as a polymers of nucleotides with four kinds of nitrogenous bases. These findings allowed to use the Feulgen method for staining of DNA from various sources of origin [10, p. 46]. Feulgen staining helps the study of the structural organization of DNA *in situ* in electron microscopy also [2, p. 345].

The Schiff's reagent in the stain specifically colors the fixed specimens DNA due to reaction with aldehyde groups that are exposed at C1 atom of deoxyribose as the result of the nitrogen bases cleavage from deoxyribose by HCl hydrolysis. Earlier in practice, a sulfite rinse followed the hydrolysis, but this is now usually not used. Light Green SF yellowish [14, p. 864] or Fast Green FCF [14, p. 618] can be used as the counterstains, providing for red DNA containing structures with light green or more deep green color backgrounds, respectively.

After dehydration in alcohol, the specimens cleared with xylene, and mounted in a resinous medium. The pink color intensity correlates with DNA content in nuclei. This is used for ploidy evaluation by microscopy and image cytometry [2, p. 345; 3, p. 1], including 3D DNA image cytometry by optical projection tomography microscopy [1, p. 017501-1] for early cancer diagnosis. By DNA quantification in Feulgen stained nuclei it is possible, for example, to identify prostatic hyperplasia and prostate cancer [15, p. 203, 3, p. 1], to confirm or exclude of malignancy in pleural effusions [11, p. 761], to detect different gynecological cancers and oral cancer [3, p. 1]. After quantitative DNA-staining, the nuclear Integrated Optical Density (IOD) is the cytometric equivalent of its DNA content. The DNA content is expressed in a "c" scale in which 1c is half the mean nuclear DNA content of cells from a normal (non-pathological) diploid population in G0/G1 cell cycle phase [3, p. 1; 17, p. 90; 18, p. 95; 20, p. 196]. The intensity color in the Feulgen reaction depends on many variables during and after the procedure. Thus, the Feulgen procedure was standardized for diagnostic DNA image cytometry [16, p. 167, 12, p. 121] with its procedure regular revisions on consensus meetings [8, p. 89].

To choose the best mounting medium for particular staining is not an easy task [6, p. 259]. From early works it is known about possible bad effects on Feulgen reaction of fixatives at particular concentrations and combinations [9, p. 276] and about preparations fading on light and in some mounting media [4, p. 301; 19, p. 179].

Aim of the study. Our study aimed i) to develop the Feulgen procedure modification that exclude the use of harsh fixation agents and mounting media and, thus, allows to escape their possible negative effects in preparations ii) to evaluate visually the effect of periodical light exposure during teaching process within 6 years onto Feulgen preparations quality.

Materials and Methods

Tissue. Human and animal tissues are less safe and more expensive than plant tissues for teaching medical and biological students. Thus, plant material was used in our experiment. The roots of onion *Allium cepa* (Linnaeus 1758) were grown hydroponically from matured bulbs in tap water until the length 5-6 cm. Then terminal root parts approximately 1 cm up from the tips were cut and stained without fixation by the method of Feulgen as described below.

The reagents were of high quality (pure for analysis).

Schiff's reagent, 1N HCl, sodium or potassium metabisulphite, freshly prepared bleaching solution (5 ml of 10% sodium metabisulphite + 5 ml of 1N HCl + 90 ml of distilled water), 45% acetic acid, mixture glycerol:water (1:1, v/v)

Preparation of Schiff's reagent.

The 0.5g of basic fuchsin were dissolve in 90 mL of boiling distilled water. After cooling to 45°C, 10 mL of 1N HCl were added slowly. When cooled to room temperature 1g of $Na_2S_2O_5$ was added (it is possible to use $K_2S_2O_5$ or metabisulfite can be substituted on sulfite, hydrosulfite or sulfurous acid). Flask content was mixed for 3 min and left in dark at room temperature for the next day until a light straw or faint pink color developed.

After that 0.5 g of fine activated charcoal was added and shaken for 3 min. The solution was filtered to a trans-

parent state and kept at 4oC in a tightly-stoppered maximum filled dark bottle in the dark.

Staining procedure.

Fresh onion root tips were transferred to distilled water through alcohol (that can be regarded as "mild fixative"): absolute ethanol 5 min, then 90%, 70%, 50%, 30% ethanol and distilled water 5 min each. Then they were placed into cool 1N HCl for 2 min then heated at 60ºC in water bath 1N HCl for 6 min. After that roots were quickly rinsed in cool 1N HCl, then in distilled water and placed in Schiff's reagent for 5 min. After that roots were rinsed in bleaching solution, then in distilled water and transferred to 45% acetic acid for 5 min. After that they were placed into drops of 45% acetic acid onto a glass slide, covered with a coverslip. Pressured preparations were made by gently pressing the coverslip against the glass slide between finger and thumb. These specimens were air dried to remove the liquid from the squashed roots and placed into a desiccator vessel with space below the platform filled by blue silica gel for further drying at dark room overnight. After that specimens were sealed with scotch tape as is demonstrated in Fig. 1 and observed in a light microscope.

Best preparations were kept at room temperature enveloped with black paper or in several layers with white paper to prevent specimen fading during 6 years.

Another set of the specimens prepared in this way was used for teaching students for 6 years with approximately 20 hours of each slide microscopy per year. At the end of six year the digital camera SIGETATM photographs of some sites in preparations were produced and compared with photos of these sites made immediately after slides preparations in year one.

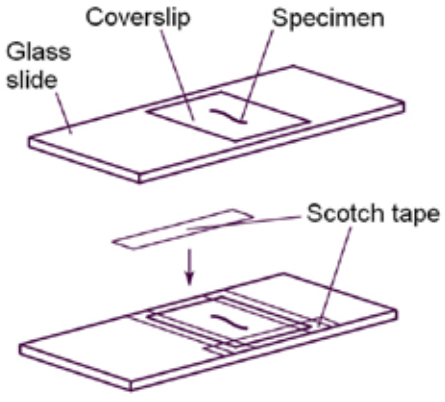


Fig. 1. Preparing the permanent specimen without mounting medium. Specimen air-dried between glass slide and coverslip is sealed on the perimeter of coverslip with strips of scotch tape of no more than 5 mm in width

Results and Discussion

Onion root tip specimens sealed on the perimeter of coverslip with strips of scotch tape of no more than 5 mm in width demonstrated no dye color deterioration, no condensate formation and no mechanical damage during a period of six years of use or storage in paper envelope.

A microscope slides with Feulgen-stained squashed onion root tips preparations that were stored and those which were in use for teaching showed no visual difference in color intensity of nuclei and cytoplasm of the cells also at start and end of the 6 years period.

Bright cytoplasm and deep rose or dark pink nuclei were observed with light microscopy in six years old preparations at the same field of views that were observed in them 6 years ago. Visual comparison of pink color intensity in the tissue and nuclei of the areas of observation suggested no visible differences in quality of photos of preparations made at the start of the 6-years period and at its end (Fig. 2). There were also no visual differences in color intensity of the slides enveloped in paper stored without use and slides used in the teaching process.

The light and mounting medium could enhance the fading of the Feulgen stained preparations [4, p. 301]. Harsh fixatives could also affect the quality of specimen preparation. Thus, we did not use both of these in our Feulgen preparations.

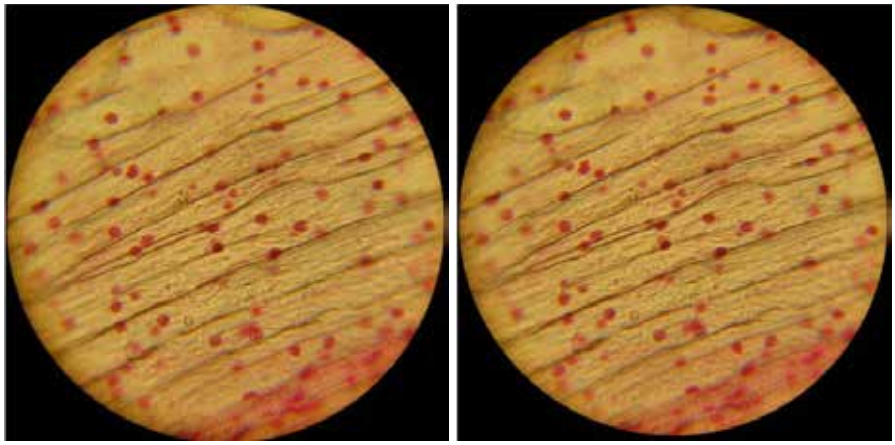
It was found that storage of Feulgen preparation of plant tissues during about one years even in the dark causes decay in dye content in the range of 5% [7, p. 294]. Precise level of fading in our preparation visually was not estimated. However, we hope that it should be less than in this work as we and our students can't detect visually 'on eye' the difference in color intensity in microphotographs of preparations made at the start and end of the 6-year period of experiment.

The observation of difference in nuclei color in same field of view, possibly, due to different stages of the cell cycle before and after S phase as intensity of color or its density is directly proportional to the amount of DNA present [2, p. 345; 11, p. 761; 13, p. 603; 16, p.167; 17, p. 140).

We prepared dry specimens without a mounting medium, with almost no air between coverslip and glass slide and with no air or humidity penetration from the surroundings. Thus, all chemical reactions in our preparations are suppressed. Use of 45% acetic acid at the final step of the specimen preparation and slide drying prevent any destructive microbe development and damage. Even if some decay in dye occurs, we can not detect it in a light microscope.

Our preparations were successfully used for 6 years and are in use now for the teaching process of medical and biological students.

We hope that our variation of the Feulgen procedure can be applied for routine analysis also.



A **B**

Fig 2. Cells of onion squashed root tip prepared by Feulgen method with use of scotch tape sealing instead of mounting medium. No significant difference seen in quality of specimen pictures at the start of the 6-years period (A) and at its end (B). Magnification 400 ×

Conclusions. Feulgen staining with use of specimen drying in air and sealing by scotch tape can be used as a cheap, reliable alternative for mounting medium application. It has no harmful effects on stained samples and provides satisfactory quality of the prepared slides for microscopic evaluation.

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