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METHOD OF STAINING SLIDES AND
STAINING SOLUTIONS THEREFOR

Andrés Ferrari, Jr., New York, N. Y., assignor, by
mesne assignments, to Technicon Chemical
Company, Inc., New York, N. Y., a corporation
of New York

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1

This invention relates to the staining of histological tissue specimens, usually referred to as slides.

The preparation of tissue to enable the microscopic examination thereof involves a plurality of treatments of the tissue prior to the cutting of the sections from the tissue specimens for the staining and mounting of the sections on the microscope slides. More particularly, in the preparation of the tissue it is necessary to immerse the tissue successively in a plurality of liquid agents for certain lengths of time, first to fix the tissue, then to wash the same for removing the fixative, then to dehydrate the tissue, usually by immersion of the tissue successively in a plurality of alcohols or other dehydration agents, then to immerse the tissue in a clearing agent, and thereafter to infiltrate the tissue with an infiltration agent such as, for example, paraffin, celloidin, etc.. After the tissue is thus treated, it is cut into sections of the desired thickness; then the paraffin or other infiltration medium is removed from said sections, usually by a solvent for the paraffin, after which the sections are mounted on the slides and stained.

The present invention is concerned with the staining of the sections, which as indicated above is performed after the paraffin is removed from said sections, and usually when the deparaffined sections are mounted on the slides.

The primary purpose of the present invention is the provision of staining solutions which are characterized by chemical stability; selectivity and specificity in staining; dependability of results without differentiation, i. e., without over-staining and destaining; and standardization of characteristics, i. e., the characteristics of the solutions can be duplicated at will in different batches, prepared at different times, and said characteristics remain constant throughout the life of the solutions.

By "selectivity" is meant the ability to stain the different cellular constituents of the tissue without any assistance by or through differentiation. For example, the selective staining of parietal and zymogenic cells, of the gastric mucosa.

By "specificity" is meant the ability to demonstrate quantitative changes of the basophilic and acidophilic qualities of cells consistently, and to do so unassisted, under varied conditions of fixation. That is to say, it must serve as a quantitative indicator of basophilic and acidophilic conditions.

The stain customarily used for the basophilic materials of animal, ordinarily human, tissue sections is known as hematoxylin. The staining solution of the present invention is of this character, but differs from known solutions in the respects required to accomplish the above mentioned objects. In particular, the staining solu-

2

tion of the present invention comprises the staining medium, namely oxidized hematoxylin, and a mordant in quantities which correspond, to the equivalent chemical combining weights of said staining medium and mordant, respectively, differing in this respect from previously known solutions in which the quantity of mordant is from twelve to twenty-five times that of the staining medium. Also, the staining solution of the present invention includes an anti-oxidant which inhibits further oxidation of the staining medium following the oxidation of the hematoxylin to produce said medium. The anti-oxidant is preferably also a vehicle or solvent for the previously oxidized hematoxylin. Further, the staining solution prepared in accordance with this invention includes a buffer system to maintain the solution at pH value of 3.0, as a result of which the staining medium is taken up readily by the tissue. I have determined also that this particular pH value of 3 is that at which the staining solution keeps best, i. e. without deterioration, for use as needed, over a period of time, so that it is unnecessary to make up fresh staining solutions at frequent intervals.

It will be understood that after the tissue is immersed in the staining solution for the usual length of time in accordance with known practice, the tissue is thereafter washed with distilled water to remove excess solution, namely solution not taken up by the tissue, and then the tissue is immersed in a reducing bath, usually lithium carbonate (Li_2CO_3), to develop the color of the stain on the basophilic material of the tissue.

As a preferred example of the method of preparing a staining solution in accordance with the present invention, the following list of substances are used in the quantities specified:

40	Hematoxylin, $\text{C}_{16}\text{H}_{14}\text{O}_6 \cdot 3\text{H}_2\text{O}$ (M. W. 356.16)—0.015 mole_____grams_____	5.3527
	Aluminum potassium sulfate, $\text{AlK}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$ (M. W. 474.37)—0.0075 mole_____grams_____	3.5577
45	Potassium permanganate KMnO_4 (M. W. 158.03)—0.01 mole_____grams_____	1.5803
	Diethylene glycol, $\text{C}_4\text{H}_{10}\text{O}_3$ _____mls_____	250.0000
	Potassium acid phthalate, $\text{KHC}_8\text{H}_4\text{O}_4$ (M. W. 204.2)—0.05 mole_____grams_____	10.2100
50	Acid hydrochloric, HCl 1/N solution_____mls_____	69.7376
	Distilled water, H_2O , sufficient to make up volume of_____mls_____	1000.0000

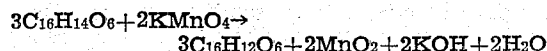
55 Of the above substance, the aluminum potassium sulfate is the mordant, and it will be understood that other suitable mordants, such as chromium trioxide and any of the mordants heretofore used, may be used instead bearing in mind, however, that the quantity of such other mordant should be adjusted in its accordance with its

3

chemical activity in relation to the chemical activity of the staining medium; the potassium permanganate is the substance used for oxidizing the hematoxylin instantly into hematein and hematoxylinic acid, and if desired any other suitable oxidizing agent, for example sodium potassium permanganate may be used instead; the diethylene glycol is a solvent for the staining medium and is also an anti-oxidant, and further acts to dissolve the products of oxidation which are formed during the preparation of the staining solution; the potassium acid phthalate and the hydrochloric acid are the buffer-forming substances and may be replaced by any other suitable buffer-forming substances; and the distilled water is the vehicle for all of the other substances of the solution, and should be as pure as possible. Freshly prepared distilled water is preferred, but if it had been prepared a few days before being used, it should be boiled to expel CO₂ which it might have absorbed.

It is important to note that in the staining solution of this invention the hematein (C₁₆H₁₂O₆) and mordant (e. g. AlK(SO₄)₂·12H₂O) are present in chemically equivalent amounts so that the activity of one is equal to that of the other. As a result of this equalization of the activities of these two ingredients two things are brought about; (1) selectivity and specificity, and (2) stability of the mordant in the solution and, as a result, elimination of the formation of precipitate. The stability of the mordant provides uniform mordanting action throughout the life of the dye solution, which is not the case with solutions which are subject to continued precipitation of the mordant.

The oxidizing agent, potassium permanganate (KMnO₄), is added in the ratio of 2 M of KMnO₄ for every 3 M of C₁₆H₁₄O₆. This ratio satisfies the equation;



Because all the available hematoxylin is converted into hematein (C₁₆H₁₂O₆), the staining power of the staining solution is at its maximum for the quantity of stain employed. Consistency of staining is achieved and maintained because no hematoxylin remains to be converted into hematein as a result of slow oxidation as occurs in the usual formulas. Since the hematein is the substance which accomplishes the staining action, this complete conversion of the hematoxylin into hematein in each batch of solution is one of the contributing factors to the standardization of the staining solution and its staining characteristics. Moreover full conversion of the hematoxylin into hematein precludes the formation of surface oxidation or scum.

Potassium permanganate was chosen as the oxidant because unlike various other oxidants it does not possess mordanting capabilities. The use of an oxidant which contributes mordanting action additional to that of the mordant already incorporated disturbs the delicate balance of this function.

It was found that the addition of a non-volatile solvent for the staining solution greatly enhances its characteristics, and that a hygroscopic action facilitates penetration of the staining solution into the tissue elements. The formation of surface scum other than that due to oxidation was precluded because the hygroscopic action prevented a concentration of the staining solution at the surface. Diethylene glycol was

4

found to be a solvent which fully satisfies the above requirements. Moreover, because of its above mentioned qualities, diethylene glycol possesses anti-oxidizing action in the staining solution.

The incorporation of buffers has two important functions; namely, (1) maintenance of correct pH for solution stability, and (2) the provision of the correct pH for optimum staining activity. It must not be concluded, however, that the correct pH alone is sufficient; correct ionic concentration at a given pH is also important. If the concentration of the buffers is too great, the chemical constituents of the tissues are not able to react properly with the staining solution. The previous remarks are important because hematoxylin solutions do not act on the tissues in the final colour that is desired, but must be reduced into an insoluble lake of the desired colour, which will in turn be determined by the mordant employed since hematoxylin is a poly-genetic stain (mordant stain). Therefore the correct pH and the correct ionic concentration of the buffered solutions are essential to consistent reduction into a constant colour value. The reduction is brought about by an alkali or constant value rather than using tap waters the alkalinity of which may vary.

The vehicle employed is distilled water, because it is a mutual solvent for all the ingredients, and because it is the one in which ionic motility can be better promoted.

In preparing the staining solution according to the above example, the hematoxylin and the aluminum potassium sulfate are dissolved separately in small quantities of distilled water and then the two solutions are mixed together and with distilled water to make 300 mls. and this mixture is boiled for about 20 minutes and then set aside to cool. For convenience in reference this solution will be called solution A. The potassium permanganate is dissolved in distilled water to a volume of about 150 mls. and this solution is then added to solution A slowly with constant stirring of the latter. This results in oxidation of the hematoxylin to produce the staining medium, as stated above. For convenience in reference this last mentioned solution will be called solution B. Then the diethylene glycol is added slowly to solution B while stirring of the same. For convenience in reference the solution B containing the anti-oxidant and solvent, diethylene glycol, will be called solution C. The buffer forming substances, namely potassium acid phthalate and hydrochloric acid are then added in succession to solution C, slowly and while stirring the potassium acid phthalate having first been dissolved in distilled water. Then after the solution is cooled to room temperature, sufficient distilled water is added to provide a total volume of 1000.00 mls. Stirring of this solution for about one hour is recommended. This is the final hematoxylin staining solution.

It will be understood that following the staining of the basophilic material of the slide, the tissue is stained with another staining solution, usually a solution of eosin for staining the acidophilic material or cytoplasm. As in the case of the hematoxylin formulas, those for eosine stain solutions have been little changed since first used in histological techniques. The stain concentration in the stain solutions in use at present calls for a concentration of 0.5% to 1.0%, and it is not uncommon to find yet greater concentrations. The usual underlying thought is

that a large quantity of stain is necessary in order to produce a strong staining effect. Such reasoning however is fallacious because proper staining cannot be achieved unless performed under proper conditions.

In accordance with the present invention, the eosin solution includes a mordanting agent, as well as buffers. More particularly, a combination of mordants each with different activities or effects and capable of producing a double color lake are used. Calcium acetate, $\text{Ca}(\text{CH}_3\text{COO})_2$, H_2O and chromium trioxide, CrO_3 are preferred, but other suitable mordants may be used. The provision of the two mordants results in the formation of a double color lake, which fastens the stain to the tissue more securely. The calcium acetate acts as a mordant in the first phase, i. e., condensation of the eosin molecule and the mordant molecule; and the chromium trioxide which also has a mordanting action is used principally because of its oxidizing action. The eosin solution also includes a swelling agent, preferably urea, $\text{NH}_2\text{CO.NH}_2$, to swell the tissue fibres, facilitating the penetration of the stain. Also, a wetting agent, preferably sodium dioctyl sulfosuccinate is included in the staining solution. The preferred substances in the buffer system are potassium phosphate monobasic and sodium phosphate dibasic, the pH of the eosin solution being 6.

The following is the preferred example of the eosin staining solution of the present invention:

Eosine (sodium tetrabromofluorescein),

$\text{C}_{20}\text{H}_5\text{O}_5\text{Br}_4\text{Na}_2$ —

0.0015 mole dye purity 91%—grams— 1.0989

Calcium acetate $\text{Ca}(\text{CH}_3\text{COO})_2 \cdot \text{H}_2\text{O}$,

0.00075 mole—grams— 0.1321

Chromium trioxide (chromic acid,

chromic anhydride), CrO_3 , 0.00075

mole—grams— 0.0750

Potassium phosphate monobasic,

KH_2PO_4 , 0.01 mole—grams— 1.3620

Sodium phosphate dibasic,

$\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ —do— 0.6166

Urea $\text{NH}_2\text{CO.NH}_2$ —do— 6.0000

Sodium dioctyl sulfosuccinate, 5%

aqueous solution—ml— 1.0000

Distilled water sufficient to make a

volume of—ml— 1000.0000

The solution of the above is prepared by dissolving the substances separately in small amounts of distilled water. The eosin is boiled gently for about twenty-five minutes, distilled water being added to compensate for evaporation but not beyond a volume of 150 mls. Then the calcium acetate solution is added, and boiling is continued for two hours, distilled water being added, if necessary, to maintain volume of 150 mls. Then the solution is allowed to cool to 60° C., and the chromium trioxide is added slowly while stirring. After this solution has been allowed to stand for about one hour, the potassium phosphate monobasic is added, while stirring. Then the sodium phosphate dibasic is added, while stirring. Then the solution is allowed to stand and cool to temperature of 40° C., when the urea and the sodium dioctyl sulfosuccinate are added in succession, in the order named, while stirring. Finally, when the solution reaches room temperature, sufficient distilled water is added to make up a volume of 1000 mls.

The removal of the paraffin and the staining of the tissue is preferably performed by an automatic immersion apparatus sold under the trade-

mark "Autotechnicon," such apparatus being shown by U. S. Patent No. 2,341,198 and other patents granted to Edwin C. Weiskopf. Heretofore, as explained in my application Ser. No. 6,822, filed February 6, 1948, now abandoned, in treating the tissue to remove the paraffin and stain the tissue, alcohols, such as ethyl alcohol, were employed for removing the xylene used as a solvent for the paraffin, and for removing the excess eosin stain from the tissue. The alcohols are objectionable because, among other things, they have a de-staining action, i. e., they apparently possess the chemical activity of removing some of the acidophilic stain, and in some instances, the basophilic from the tissue. Further, the alcohols are hygroscopic and thus by absorbing water from the atmosphere, become diluted. Moreover, the alcohols evaporate rapidly at room temperature so that the slides, i. e., the tissue sections which are mounted on the glass slides, may dry more or less during their transfer from one liquid to another during the operation of the automatic immersion apparatus. As stated in my said application, Ser. No. 6,822, these objections are obviated by substituting for the alcohols a solvent which is capable of removing the xylene and the excess stain, i. e., the stain which has not become attached to the tissue, but without de-staining the tissue. This solvent is also characterized by a high boiling point so that it does not evaporate at room temperature, and it is completely soluble in water, alcohols, and aromatic hydrocarbons. More specifically, in accordance with the present invention I use diethylene glycol monoethyl ether acetate,

$\text{CH}_3\text{COOCH}_2\text{CH}_2\text{OCH}_2\text{CH}_2\text{OC}_2\text{H}_5$

as the solvent. This solvent has a boiling point of 217.7°C; and, as stated it is soluble in water, and is non-hygroscopic, although it is a solvent for water.

The following is an example of the preferred technique employed in removing the paraffin from the tissue sections and in staining them. For convenience in reference the tissue section will be referred to as slides which is the term used by pathologists and their technicians. Also for convenience, the diethylene glycol monoethyl ether acetate will be designated as "solvent F." The method of the present invention starts with the slides having the paraffin therein, and the immersion operations are performed automatically on the "Autotechnicon," having twelve beakers containing the liquids referred to, respectively. The beakers are arranged in the positions indicated and contain the liquids specified, and the slides are immersed in said liquids for the indicated periods of time, in minutes, as follows:

Position on Machine	Tissue treating Agent	Time
		Minutes
1-----	Xylene, pure-----	2
2-----	Solvent F, pure (for tissues fixed with Mercuric Chloride, add Iodine 0.25%)-----	2
3-----	Solvent F, pure-----	2
4-----	Distilled Water-----	2
5-----	Hematoxyline Solution-----	5
6-----	Distilled Water-----	4
7-----	Lithium Carbonate 0.01 N-----	2
8-----	Distilled water-----	4
9-----	Eosine Solution-----	5
10-----	Solvent F, pure-----	5
11-----	Solvent F, pure-----	5
12-----	Solvent F, pure-----	5
	Remove from the machine and place in a beaker of Xylene for-----	5

The solutions should be changed preferably as follows:

- a. Xylenes, every week.
- b. Solvent F, 2nd, 3rd, and 4th place every 10-20 days.
- c. Distilled water, 6th and 8th place every rack of slides.
- d. Lithium carbonate, every 2 weeks.
- e. Solvent F, 10th, 11th and 12th place, every 2 weeks discarding the 10th place sol. and moving the 12th and 11th down, then replacing the 12th place with fresh solution.
- f. The dye solutions will last approximately four months in constant use without requiring any attention whatsoever.

The actual time that these reagents may be employed will, in practice, be dictated by the amount of work that is being performed daily in the laboratory.

It will be understood that the use of the above solvent F is preferred but that the use of the staining solutions of the present invention are not limited to methods involving the use of that particular solvent.

It will be understood that the invention is not limited to the examples hereinbefore set forth or to the other specific aspects, except as may be required by the appended claims.

Having thus described my invention, what I claim and desire to secure by Letters Patent is:

1. A solution for staining the basophilic material of animal-tissue for microscopic examination thereof, comprising a staining medium formed by oxidizing hematoxylin, a mordant for said staining medium, said staining medium and mordant being present in the solution in equivalent chemical combining weights of the staining medium and mordant, respectively, and a buffer system in said solution to maintain the same in an acidic state and having an ionic concentration favorable to staining the tissue and maintaining the solution in a stable condition.

2. A solution for staining the basophilic material of animal-tissue for microscopic examination thereof, comprising a medium consisting of hematein, a mordant for said staining medium, said staining medium and mordant being present in the solution in equivalent chemical combining weights of the staining medium and mordant, a buffer system in said solution to maintain the same in an acidic state and having an ionic concentration favorable to staining the tissue and maintaining the solution in a stable condition, and an anti-oxidant in said solution.

3. A solution for staining the basophilic material of animal-tissue for microscopic examination thereof, comprising a staining medium consisting of hematein, a mordant for said staining medium being present in the solution in equivalent chemical combining weights of the staining medium and mordant, respectively, a buffer system in said solution to maintain said solution at a pH of about 3 and having an ionic concentration favorable to staining the tissue and maintaining the solution in a stable condition, and an anti-oxidant in said solution.

4. A solution for staining the basophilic material of animal-tissue for microscopic examination thereof, comprising a staining medium consisting of hematein, a mordant for said staining medium, said staining medium and mordant being present in equivalent chemical combining weights of the staining medium and mordant, respectively, and a buffer to maintain said solution at a pH of about 3 and having an ionic concen-

tration favorable to staining the tissue and maintaining the solution in a stable condition.

5. A solution for staining the basophilic material of animal-tissue for microscopic examination thereof, comprising a staining medium formed by oxidizing hematoxylin, a mordant for said staining medium, said staining medium and mordant being present in the solution in equivalent chemical combining weights of the staining medium and mordant, respectively, a buffer system in said solution to maintain the same in an acidic state and having an ionic concentration favorable to staining the tissue and maintaining the solution in a stable condition, and diethylene glycol in an amount sufficient to dissolve the products of oxidation formed in said oxidation of the hematoxylin and to prevent subsequent oxidation of the solution.

6. The method of staining the basophilic material of animal-tissue slides for microscopic examination, comprising applying to the tissue section a solution of a staining medium formed by oxidizing hematoxylin, a mordant for said staining medium, said staining medium and mordant being present in equivalent chemical combining weights, and a buffer system to maintain the same in an acidic state and having an ionic concentration favorable to staining the tissue and maintaining the solution in a stable condition.

7. The method of staining the basophilic material of animal-tissue slides for microscopic examination, comprising applying to the tissue section a solution of a staining medium formed by oxidizing hematoxylin, a mordant for said staining medium, said staining medium and mordant being present in equivalent chemical combining weights of the staining medium and mordant, respectively, and a buffer in said solution to maintain at a pH value of about 3 and having an ionic concentration favorable to staining the tissue and maintaining the solution in a stable condition.

8. The method of staining the basophilic material of animal-tissue slides for microscopic examination, comprising applying to the tissue section a solution of a staining medium formed by oxidizing hematoxylin, a mordant for said staining medium, said staining medium and mordant being present in the solution in equivalent chemical combining weights of the staining medium and mordant, respectively, said solution also containing a buffer system having a pH value of about 3, and having an ionic concentration favorable to staining the tissue and maintaining the solution in a stable condition and diethylene glycol in an amount sufficient to dissolve the products of oxidation formed in said oxidation of the hematoxylin and to prevent subsequent oxidation of the solution.

9. The method of staining animal-tissue for microscopic examination, comprising applying to the tissue a solution of a staining medium formed by oxidizing hematoxylin, a mordant for said staining medium, said staining medium and mordant being present in the solution in equivalent chemical combining weights of the staining medium and mordant, respectively, for staining only the basophilic materials of the tissue, said solution also containing a buffer system to maintain the same in an acidic state and having an ionic concentration favorable to staining the tissue and maintaining the solution in a stable condition, then treating the tissue with a reducing agent to develop the color of the stain on the tissue, and then applying to said tissue an eosin solution to stain the acidophilic material of the

tissue, said eosin solution containing a mordant in amount substantially the chemical equivalent of the amount of eosin present, and a buffer in amount to impart to said eosin solution a pH of about 6 and having an ionic concentration favorable to staining the tissue and maintaining the solution in a stable condition.

10. A solution for staining the acidophilic material of animal-tissue for the microscopic examination thereof, comprising a staining medium consisting of eosin, a mordanting agent, said staining medium and mordanting agent being present in the solution in equivalent chemical combining weights, and a buffer system to maintain the same in an acidic state and having an ionic concentration favorable to staining the tissue and maintaining the solution in a stable condition.

11. A solution for staining the acidophilic material of animal-tissue for the microscopic examination thereof, comprising a staining medium consisting of eosin, a mordanting agent, said staining medium and mordanting agent being present in the solution in equivalent chemical combining weights, and a buffer system to maintain the same in an acidic state and having an ionic concentration favorable to staining the tissue and maintaining the solution in a stable condition, said mordanting agent comprising a combination of mordants forming a double color lake, and a buffer system to maintain the same in an acidic state and having an ionic concentration favorable to staining the tissue and maintaining the solution in a stable condition.

12. A solution for staining the acidophilic material of animal-tissue for microscopic examination thereof, comprising a solution of eosin, a mordant, and a buffer system to maintain said solution at a pH of about 6, and having an ionic concentration favorable to staining the tissue and maintaining the solution in a stable condition, said staining medium and mordanting agent being present in the solution in equivalent chemical combining weights.

13. A solution, for staining the basophilic material of animal tissue for microscopic examination thereof, made by mixing distilled water, hematoxylin and potassium permanganate in the proportion of 3 mols of hematoxlin for each 3 mols of potassium permanganate, an aluminum potassium sulfate mordant in the proportion of one mol of the mordant for each 2 mols of hematoxylin, diethylene glycol in amount sufficient to dissolve the products of oxidation formed by the oxidation of the hematoxylin by the potassium permanganate and to prevent subsequent oxidation of the solution, and potassium acid phthalate and hydrochloric acid, the latter two constituents being present in amount to maintain the pH of said solution at about 3 and having an ionic concentration favorable to staining the tissue and maintaining the solution in a stable condition.

14. A solution, for staining the acidophilic material of animal tissue for microscopic examina-

tion thereof, made by mixing distilled water, eosin, calcium acetate, chromium trioxide, the latter three constituents being present in the proportion of one mol of calcium acetate and one mol of chromium trioxide for each 2 mols of eosin, alkali metal phosphates in amount to maintain the solution at a pH of about 6 and having an ionic concentration favorable to staining the tissue and maintaining the solution in a stable condition, a tissue swelling agent and a wetting agent.

15. A solution for staining the basophilic material of animal-tissue for microscopic examination thereof, comprising diethylene glycol, a staining medium consisting of hematein, a mordant for said staining medium from the group consisting of aluminum potassium sulfate and chromium trioxide, said staining medium and mordant being present in the solution in equivalent chemical combining weights of the staining medium and mordant, respectively, and a buffer system in said solution for maintaining said solution at a pH of about 3, and having an ionic concentration favorable to staining the tissue and maintaining the solution in a stable condition.

16. A solution, for staining the basophilic material of animal tissue for microscopic examination thereof, made by mixing distilled water, hematoxylin and potassium permanganate in the proportion of 3 mols of hematoxylin for each 2 mols of potassium permanganate, a chromium trioxide mordant in the proportion of one mol of the mordant for each 2 mols of hematoxylin, diethylene glycol in amount sufficient to dissolve the products of oxidation formed by the oxidation of the hematoxylin by the potassium permanganate and to prevent subsequent oxidation of the solution, and potassium acid phthalate and hydrochloric acid, the latter two constituents being present in amount to maintain the pH of said solution at about 3, and having an ionic concentration favorable to staining the tissue and maintaining the solution in a stable condition.

ANDRÉS FERRARI, JR.

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