The method described has the advantage that the surface of contact between disinfectant and medium is considerably increased and shows better the evenness of action than is the case with the agar cup method. It also imitates the use of thin layers as applied for practical purposes.

Rapid Staining Methods in Plant Histology

By Robert S. McLean and Edward J. Ireland†*

The routine methods for the differential staining of plant tissue for histological study in use to-day require from three to twenty-four hours or more. McClung (1929) and Chamberlain (1932) recommend for routine work a primary stain of Safranin for three to twenty-four hours at room temperature, and a counterstain of hæmatoxylin for one-half to six hours. Chamberlain (1932) recommends six hours or more for a primary stain of Malachite Green or Methylene Blue, and one-half to one minute for counterstaining with Eosin or Erythrosin. These procedures are typical with respect to the time required to obtain an effective differential stain.

Obersteiner (1878) used a solution of carmine over a bath of boiling water for staining nerve cells. Bacteriologists use heat for staining bacterial spores and such organisms as *Mycobacterium tuberculosis* which "take" a stain slowly. By the use of heat in the "acid-fast" technique, the tuberculosis organism is stained in four or five minutes where twelve to twenty-four hours at room temperature are required.

Since heat is applicable in bacteriological staining, it seemed logical to assume that it would also be effective in botanical staining. Therefore the use of heat was investigated and successfully used in the following methods.

EXPERIMENTAL

To avoid the numerous transfers through increasing and decreasing concentrations of the alcohol, 95 per cent ethyl alcohol was used as the solvent for the stains in all cases except for one of the counterstains, Methyl Blue. For this stain, 80 per cent ethyl alcohol was used since the dye was very slightly soluble in the 95 per cent alcohol. The few stains which have a standardized formula were prepared with the directed solvent.

Paraffin sections of ten to twenty microns thickness fixed to slides with Mayer's albumin fixative were used throughout the series of experiments.

METHOD I

The first method which gave consistently good differentiation and showed considerable saving of time was conducted as follows:

1. Two to four drops of the primary stain (or enough to cover the sections) were placed on the slide, the solvent ignited and allowed to burn off completely.
2. The slide was washed briefly but thoroughly in 95 per cent ethyl alcohol. This was best accomplished by agitating the slide in the alcohol contained in a deep beaker or Coplin jar. The washing removed the excess stain from the slide and partially removed the primary stain from the cellulose tissue.
3. Four to six drops (or enough to flood the sections) of the counterstain were placed on the slide and allowed to act for ten to thirty seconds as required (see Table I). For the slower acting counterstains, Methyl Blue and Orange G especially, the solvent was burned off as in (1). With woody sections even the faster acting counterstains gave best results when flamed.
4. The slide was again washed in 95 per cent ethyl alcohol as in (2), cleared and examined.

Table I.—Relative Approximate Time Required for the Various Counterstains for the Above Process

Group I 5–10 Seconds	Group II 10–20 Seconds	Group III 20–30 Seconds
Erythrosin	Eosin Y	Orange G
Eosin B	Picric Acid	Methyl Blue
Orange I	Acriflavine	Methyl Red
Acid Fuchsin	Congo Corinth	
	Bismarck Brown	
	Hæmatin	

The chief difficulty encountered in this technique was the manipulation of the counterstains. The time required to obtain the proper degree of differentiation varied with the counterstain used and also with the nature of the sections, but this variation was only a matter of a few seconds. For example, Acid Fuchsin used on leaves or on herbaceous stems required only about 5 to 10 seconds, Eosin Y required 10 to 15 seconds and Methyl Blue about 20–30 seconds. On more woody sections these time ranges increased about 5 to 10 seconds. The lengths of time as classified in Table I were found to be only approximate. Since the time during which the counterstain was allowed to act was, in most cases, so very short, accurate timing was impossible.

Perhaps the best method of handling the counterstain is to flood the sections with the counterstain, allow it to act for five to ten seconds, wash briefly in

* Graduate student, School of Pharmacy, Univ. of Florida.

† Dept. of Pharmacognosy & Pharmacology, University of Florida.

the alcohol and examine the sections microscopically while still wet with the alcohol. If the desired degree of differentiation had not been obtained, the counterstain was applied again and allowed to act for five to ten seconds longer, the slide washed and examined again. This process was repeated until the proper differentiation was obtained.

Another method of handling the counterstain found to be of value in staining fairly large sections, such as cross or longitudinal sections of roots and stems measuring more than about 5 mm. in diameter or width, was as follows: The slide was held over a piece of white paper after the primary stain had been applied, the sections were flooded with a few drops of the counterstain and allowed to act until the color of the sections changed from the color of the primary stain to that of the counterstain. Then the excess stain was poured off, the slide was washed and examined. This method almost invariably stopped the action of the counterstain at the point where differentiation was good.

It was found that if the counterstain was allowed to act too long the primary stain was retracted from the lignified tissue and replaced by the counterstain as is the case in the routine staining methods. However, when this happened using this "flaming" technique the primary stain could be applied again as indicated above. The sections did not need to be decolorized before restaining.

The handling of the counterstain was easily mastered after a few attempts, the author often being able to estimate accurately the time necessary for a given counterstain to act. Usually only three or four slides were necessary to establish the time range for each counterstain.

The following stains have proved to be suitable for lignified tissue, cork and cuticle:

1.	Victoria Green	(du Pont)
2.	Malachite Green oxalate	(National Aniline & Chem. Co.)
3.	Brilliant Cresyl Blue	(National Aniline & Chem. Co.)
4.	Brilliant Green	(National Aniline & Chem. Co.)
5.	Janus Green	(National Aniline & Chem. Co.)
6.	Thionin	(National Aniline & Chem. Co.)
7.	Azure II	(National Aniline & Chem. Co.)
8.	Methylene Blue	(U. S. P. medicinal)
9.	Safranine A	(Coleman & Bell)
10.	Rhodamine B	(Coleman & Bell)
11.	Hæmatin	(Coleman & Bell)
12.	Basic Fuchsin	(Coleman & Bell)
13.	Gentian Violet	(Coleman & Bell)

Of these, Hæmatin gave somewhat poor results. This was probably due to the fact that it imparted only a very indistinct color to the tissue, and this was easily replaced by, and difficult to differentiate with, any of the counterstains. Basic Fuchsin left a rather dense film over the sections which was hard to differentiate properly.

The following have proved to be applicable as counter-stains:

1.	Erythrosin	(Coleman & Bell)
2.	Eosin Y	(Coleman & Bell)
3.	Eosin B	(Coleman & Bell)
4.	Orange G	(Coleman & Bell)
5.	Orange I	(Coleman & Bell)
6.	Acid Fuchsin	(Coleman & Bell)
7.	Congo Corinth G	(Coleman & Bell)
8.	Hæmatin	(Coleman & Bell)
9.	Methyl Blue[a]	(Coleman & Bell)
10.	Picric Acid	(Merck)
11.	Bismarck Brown	(Merck)
12.	Acriflavine	(U. S. P. medicinal)
13.	Methyl Red	(Eastman Kodak & Chem. Co.)

[a] Saturated soln. in 80 per cent ethyl alcohol; due to low solubility in 95 per cent.

Of these, Congo Corinth, Methyl Red and Hæmatin gave poor results, probably because they gave a very indistinct color, hard to obtain differentiation without retracting the primary stain, and easily removed in the final washing process.

All stains used in this method were in 1 per cent solution except Acid Fuchsin, Orange I and these dyes were less than 1 per cent soluble. Acid Fuchsin and Orange I were used in a 0.5 per cent solution due to the fact that higher concentrations acted too fast to be readily controlled. Those dyes which were less than 1 per cent soluble were used in saturated solution. As stated above, 95 per cent ethyl alcohol was the solvent throughout, except for Methyl Blue which was dissolved in 80 per cent ethyl alcohol due to poor solubility in the 95 per cent.

Perhaps the best combinations of stains observed for use on leaf sections were Methylene Blue counterstained with Eosin or Erythrosin; Victoria Green with Picric Acid; and Safranin A or Rhodamine B with Methyl Blue. The best combinations observed for use on stem and root sections were: Methylene Blue counterstained with Eosin, Erythrosin or Orange G; Malachite Green with Acid Fuchsin or Orange I; and Safranin A with Methyl Blue.

METHOD II

The next step which seemed logical in the investigations of possible rapid staining methods was to attempt to combine two stains, a primary and a counterstain in a single solution. This would further reduce the time necessary to obtain an effective differential stain. After many attempts, this was accomplished, thereby obtaining a good differential stain using only two simple operations, and reducing the time to about fifteen to thirty seconds for the complete operation.

The solution used in this double stain technique was prepared by mixing a solution of a primary with one of the counterstains used in Method I. The proportions were varied until a satisfactory mixture was found. The first combinations found indicated that approximately 10 parts of the counterstain were needed for 1 part of the primary stain. Other combinations indicated that while this proportion was generally true, there was a variation necessary for some pairs of stains. Later it was found that

some of the more common double stains used in blood stainjng were also applicable to the differential staining of plant tissue. Wright's, Giemsa's and Jenner's stains were the ones tried, and these were satisfactory.

The technique in this method was identical with Steps 1 and 2 of Method I; the sections were covered with the mixture of the stains, the solvent ignited and allowed to burn off, the slide washed briefly but thoroughly in 95 per cent ethyl alcohol.

In this technique, it was found that the time during which the sections were washed varied with the various stain combinations and with the nature of the sections. With some stain, woody sections required a longer washing process than did herbaceous sections. Generally, the time for the washing process is about five to fifteen seconds.

The following proved to be suitable as double stains:

1. Malachite Green (1% soln. in 95% ethyl alcohol) 0.1 cc.
   Acid Fuchsin (0.5% soln. in 95% ethyl alcohol) 1.0 cc.
2. Victoria Green (1% soln. in 95% ethyl alcohol 0.1 cc.
   Acid Fuchsin (0.5% soln. in 95% ethyl alcohol 1.0 cc.
3. Basic Fuchsin (1% soln. in 95% ethyl alcohol) 0.1 cc.
   Methyl Blue (satd. soln. in 80% ethyl alcohol) 1.0 cc.
4. Safranin A (1% soln. in 95% ethyl alcohol) 0.3 cc.
   Orange G (satd. soln. in 95% ethyl alcohol) 2.0 cc.
5. Wright's Stain (standardized formula) (methyl alcohol solution)
6. Giemsa's Stain (standardized formula)
7. Jenner's Stain (powder, satd. soln. in 95% ethyl alcohol)
8. Wright's Stain (powder, satd. soln. in 95% ethyl alcohol)
9. Azure II—Eosin[a] (powder, satd. soln. in 95% ethyl alcohol)
10. Methylene Blue (1% soln. in 95% ethyl alcohol) 0.2 cc.
    Acid Fuchsin (0.5% soln. in 95% ethyl alcohol) 1.0 cc.
11. Methylene Blue (1% soln. in 95% ethyl alcohol) 0.3 cc.
    Erythrosin (satd. soln. in 95% ethyl alcohol) 2.0 cc.
12. Methylene Blue[b] (1% soln. in 95% ethyl alcohol) 0.3 cc.
    Orange G[b] (satd. soln. in 95% ethyl alcohol) 1.0 cc.

[a] National Aniline & Chem. Co.
[b] This stain showed a green precipitate within a few minutes after mixing. Good results were obtained with this combination, but due to the formation of this precipitate it was discarded.

Giemsa's Stain, being a solution in equal parts of methyl alcohol and glycerin, required somewhat different handling than the others. Only half of the solvent was volatile so when the solution was ignited the methyl alcohol burned off readily but the glycerin began to bubble and "splutter" as soon as the methyl alcohol had burned off. It was found that if the flame was blown out as soon as the glycerin began to bubble, the slide washed and examined, the sections showed good differentiation.

METHOD III

In the investigation of the double stains, it was found that in sections which contained cork in addition to lignified and cellulose tissue, a "triple-color" differentiation could be obtained. This was observed first in the Methylene Blue-Eosin blood stains. The Methylene Blue stained the lignified tissue blue and the cork tissue green, while the Eosin stained the cellulose tissue pink or red. Since the Methylene Blue gave two colors, it seemed that this "triple color" stain could be obtained with any double stain in which Methylene Blue was the primary stain. This was successfully proved by other combinations using Methylene Blue with three other counterstains, Acid Fuchsin, Orange G and Erythrosin.

The double stains which gave this "triple-color" differentiation were the following:

1. Wright's Stain (standardized formula)
2. Jenner's Stain (satd. soln. in 95% ethyl alcohol)
3. Wright's Stain (N. A. C. Co., powder, satd. in 95% ethyl alcohol)
4. Giemsa's Stain (standardized formula)
5. Azure II—Eosin (satd. soln. in 95% ethyl alcohol)
6. Methylene Blue (1% in 95% ethyl alcohol) 0.2 cc.
   Acid Fuchsin (0.5% in 95% ethyl alcohol) 1.0 cc.
7. Methylene Blue (1% in 95% ethyl alcohol) 0.5 cc.
   Erythrosin (satd. in 95% ethyl alcohol) 2.0 cc.
8. Methylene Blue (1% in 95% ethyl alcohol) 0.3 cc.
   Orange G (satd. in 95% ethyl alcohol) 1.0 cc.

In Giemsa's Stain and in Azure II—Eosin, the primary stain is Azure II, which is not Methylene Blue alone, but a combination of the HCl salts of Methylene Blue and Methylene Azure (Methylene Blue Sulfone).

To date, these methods have been used successfully on cross-sections of the leaves of dicotyledonous plants: *Jasimum primulinum, Cinnamomum camphora* and *Mentha citrata*, and a monocotyledonous plant, *Holcus halepensis;* on cross-and longitudinal-sections of the stems of dicotyledonous plants, *Crotalaria retusa* and *Cracca virginiana* and cross-sections of the stems of a monocotyledon, *Holcus halepensis;* on cross- and longitudinal-sections of the roots of two dicotyledonous plants, Southern smilax and *Cracca virginiana;* and cross- and longitudinal-sections of the root of a monocotyledon, *Zingiber officinale;* and on the germinating embryo of the avocado (*Persea americana*).

In more than 200 slides stained by this method there has been observed no apparent damage to the cells or their contents. In the leaf sections the

chloroplasts were apparently unchanged by the staining process. In a series of more than 30 slides of the root of smilax, the differentiation was very good and the contents of the storage parenchyma cells, such as starch grains and calcium oxalate rosettes, were apparently unchanged by the staining process.

Of twenty-seven alcohol-soluble stains used, only one—Sudan III—proved entirely unsuccessful. Those which were called "poor" in the discussion of Method I were applied with a fair degree of success on a few slides. However, they required special handling which increased the time consumed to such an extent as to render the stain of little practical value. For example, Hæmatin was used as a primary stain on several slides, but it had to be flamed on the slide several successive times without washing after application, and then counterstained with a rapidly acting stain from Group I of Table I. Likewise, Methyl Red was used as a counterstain by applying a primary stain such as Methylene Blue several times, then using the Methyl Red and flaming it on the slide several times. For this reason those stains called "poor" should be entirely discarded due to the fact that they require too much time, too much handling and they do not give as good results as others which are easily applied.

A possible disadvantage of this method was seen in the fact that each slide must be handled individually throughout the entire staining process. However, in the usual methods of staining, the slides must be given individual handling at some stage of the process even when they are stained in groups.

Another possible disadvantage of this method which was apparent was that the staining solutions were used only once. The primary stain must be flamed and occasionally the counterstain also. The washing was brief, so the counterstain was rapidly discolored by the primary stain which was partially retracted. However, if the stains were applied by means of a medicine dropper, only two to four drops were used. The concentration of the staining solutions need never be greater than 1 per cent and in most cases could probably be considerably less. For example, Methylene Blue was equally effective in 0.1 per cent and in 1 per cent solution. Malachite Green was equally effective in 0.1 per cent, 1 per cent and in 7.5 per cent (saturated) solution.

SUMMARY

This paper is presented as a preliminary report, and it is hoped that these methods (although two methods were described separately, both are dependent upon the same principle, namely, flaming the primary stain) may prove of value to other workers in the field, and also that additional information may be brought to light by further investigation.

The authors feel that the great saving of time effected by this method should attract the attention of others interested in perfecting rapid methods of differential botanical staining.

At this time the results of these investigations are applicable only to temporary mounts. The first slides stained by this method were mounted in Canada balsam October 25, 1938. Sufficient time has not elapsed for confirmation of the permanency of the stains.

It is the intention of the authors to conduct further research on this problem; to attempt to establish a more standardized technique; to investigate further the concentration of the staining solution necessary for effective differentiation, as well as to apply other stains and combinations of stains to this technique.

Announcement of a Study to Evaluate Original Serologic Tests for Syphilis

More than five years ago the Committee on Evaluation of Seriodiagnostic Tests for Syphilis, in coöperation with the United States Public Health Service, conducted a study to evaluate original serologic tests for syphilis or modifications thereof in the United States. The results of this study were published shortly after the investigation was completed.[1]

Consideration is now being given by the Committee to the organization of a second evaluation study of original serologic tests for syphilis or modifications thereof within the next year. The second evaluation will be conducted utilizing methods comparable to those employed in the first study.[2]

Serologists who have an original serologic test for syphilis or an original modification thereof and who desire to participate in the second evaluation study should submit their applications not later than October 1, 1940. The applications must be accompanied by a complete description of the technic of the author's serologic test or modification. All correspondence should be directed to the Surgeon General, United States Public Health Service, Washington, D. C.

---

[1] *Ven. Dis. Inform.*, Washington, 16 (1935), 189. *J. Am. Med. Assoc.*, Chicago, 104 (June 8, 1935), 2083.

[2] *J. Am. Med. Assoc.*, Chicago, 103 (Dec. 1, 1934), 1705.