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Introduction

The problem of evaluating the virtual image in fluorescence microscopy is essentially one of assaying the several attributes of color, viz. hue, value, and chroma or saturation. DeMent (1942) suggested that fluorescence phenomena be reported according to the recommendations on color of the American Optical Society. This is a qualitative estimate employing some twenty-one hue names qualified by eight adjectives. The present work has been designed to delimit these adjectives and to provide a common denominator for the concept of hue.

The concept of hue varies not only with an individual's inherited ability to perceive color, but also with the extent of his training in the observation of color. Many people are not color blind, but are color ignorant, which is to say that their ideas of color are not the same as those of the person who classifiies them as color ignorant. What is bright or dull, or light or dark, to one person is not necessarily the same to another. What is pink, or orange, or blue to one person may not be the same to another, at least not to the same degree. Consequently, there is a multiplicity of terms in the nomenclature of color. Because of this it is desirable to employ a color standard which takes these variations into account and to adapt its use to the problem at hand.

Literature

The hue or name of a color is described most accurately when the frequency or wavelength is specified; on the other hand the variation among the common names for any given color is considerable. Many workers have devised charts and codes to standardized the nomenclature. The Book of Color by Munsell (1929) is outstanding among these and has provided much of the impetus for the present work. However, the more modern (1950) second edition of a Dictionary of

Color by Maerz and Paul offers some advantages over that of Munsel. It comprises a single volume instead of the two of Munsel and the variables hue and chroma are incorporated in each of their charts instead of in separate charts. The quality of the inks chosen by Maerz and Paul resemble the quality of the fluorescent image, and their method of tabulation by means of rows and columns is very simple to use.

One must remember, however, that the color of inks and pigments is modified by the amount of light which they reflect, whereas the color of fluorescence is due to the color of the emitted visible light itself. Therefore the gray scale which is commonly used to measure the reflectance or value of a colored pigment is meaningless in evaluating the colors of visible light. Instead of value, one must speak of the intensity of light. That this can be readily determined in fluorescence microscopy was shown by Yos (1956) who used a photographic step tablet to determine a set extinction values for the fluorescence of a select group of plant tissues. In this way he was able to detect differences in intensity too minute for the unaided eye to perceive.

Materials and Methods

In Maerz and Paul plates #1 (red to orange), #9 (orange to yellow), #17 (yellow to green), #25 (green to blue-green), #33 (blue-green to blue), #41 (blue to red), and #49 (purple to red) all have the same value. To facilitate their use in rather cramped quarters they were copied by natural daylight on Ektachrome film. The resulting 2¼ by 3¼ inch transparencies were mounted under glass and were then placed upon an illuminated viewing box close by the microscope. A movable black mask with a circular 5 mm hole permitted a single color square to be viewed at a time. The resulting spot of light could be reflected into the eye of the microscopist in such a way that the effect was one of viewing a tissue with a small colored spot beside it and comparisons were easy to make. Due to filters within the instruments available, a camera lucida could not be employed for this. Excellent results were obtained by holding a clean glass slide or cover slip over the ocular at an acute angle to it and facing the illuminated color standard. This not only reflected the spot of light into the eye, but also permitted considerable choice in the positioning of it within the field of the microscope. The color index of each tissue was recorded and then translated back into the index of the Dictionary itself. To do this it was necessary to view the Ektachrome transparency on the same viewing box and to compare it with the original standard of Maerz and Paul. This was done by the light of a 15-watt daylight

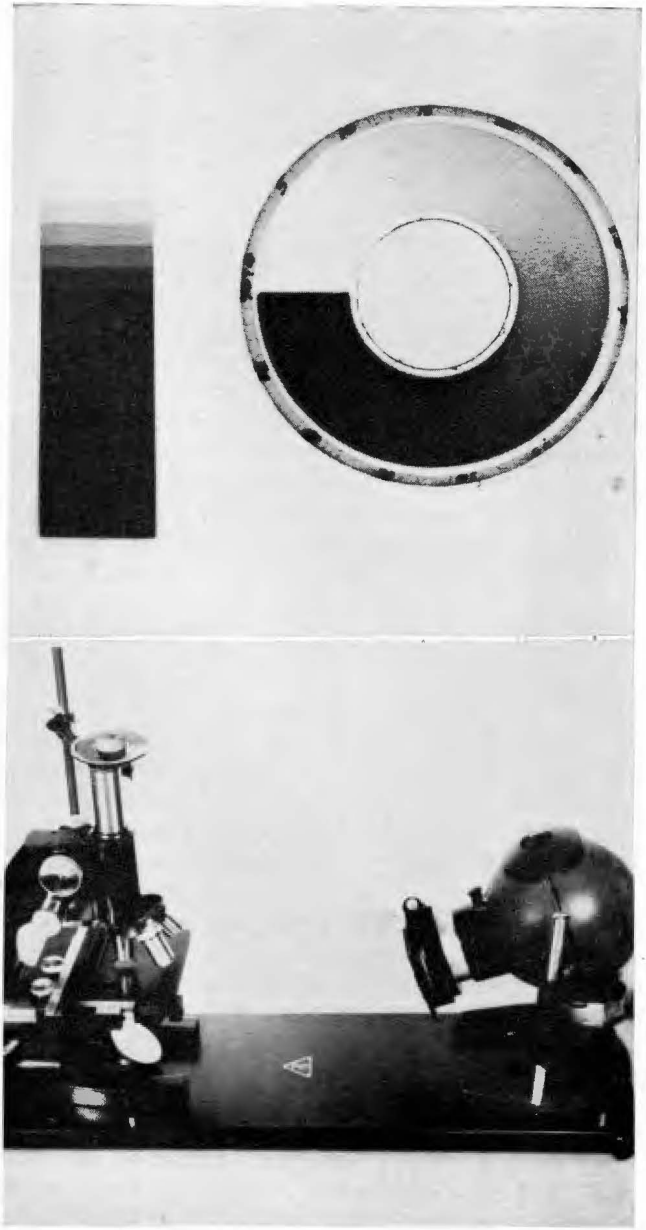

Figure Legend: See text. Figure 1 above, figure 2 below.

fluorescent desk lamp. All colors reported in this paper therefore are those of Maerz and Paul, not those of the transparency.

The step tablet used in earlier phases of this work (Yos, 1956) had a density range of 0.0 to 3.0 with a density gradient of 0.3 from step to step. In the present work this tablet was used to supplement a circular density wedge with a density range of 0.0 to 2.0. Both wedge and tablet were of the smallest standard size produced by Eastman Kodak and are shown in figure 1.

A wooden plug was driven into the large end of a one-holed #5 rubber stopper to permit clamping in in a ring stand. The circular wedge was mounted on the inverted stopper and in this way was supported over the microscope as in figure 2. The image of the exit pupil of the microscope served as an index to mark the position of the wedge which was provided with an arbitrary scale of seventy units about its periphery. These scalar units were marked at five degree intervals. The scale was then calibrated with a densitometer. In use, the wedge was rotated so that the dense sector passed over the ocular first and the point at which cellular details became barely visible was recorded. The density of the wedge or tablet-wedge combination at each of these points is referred to below as the intensity value. The intensity values were established as density steps of 0.1 and were recorded as the upper limit of the step in which the reading fell.

To test the method, the same source of ultra violet light used previously was used again, but was clamped to a Bausch and Lomb baseboard with the lamp base (socket) 15 inches from the aluminized mirror of the microscope. The top of the condenser was removed and a dark field stop was added. Corning filters #5840 and Noviol-O were used at the lamp and in the ocular respectively and all readings were made at a magnification of 100 X. 0.1% aqueous solutions of acridine orange, rhodamine red, and titian yellow were used to stain paraffine sections of stems of *Ricinus, Coleus,* and geranium which had been killed in F.A.A. Each section was stained for 30 minutes and was then washed 5 minutes each in water, 50, 75, 95, and 100% alcohol. After an additional 5 minutes in xylol they were mounted in isobutyl methacrylate. Five tissues were selected for examination.

Results

Epidermis, cork, cortex, xylem, and pith are recorded in each of the tables below. Table 1 lists their fluorescence with acridine orange, the best stain of the three; table 2 lists their fluorescence with rhodamine red, the most variable stain of the three; table 3 lists their fluorescence with titian yellow, the stain producing the best microscopic definition but the least differentiation. The tabulations are based upon this formula: Intensity/Color Index. For example,

the epidermis of Coleus stained with acridine orange is given as 1.1/1L6. This means that the fluorescence was strong enough to be visible through a density of 1.1 and that a reasonably close match of its red color can be found in Plate -1 of Maerz and Paul in Row - 6 of Column - L. The color limits of the seven plates chosen for this study are listed above.

Table 1

(Fluorescence with acridine orange)

	Coleus	Ricinus	Geranium
Epidermis	1.1/1L6	1.5/1L5	1.3/1L9
Cork	1.5/33I5	1.5/33B5	1.8/9L10
Cortex	1.5/1L6	1.5/1L5	1.3/1L8
Xylem	1.8/9J7	2.1/9K7	1.9/9L11
Pith	Not observed (hollow stem)	1.7/1L11	1.2/1L9

Table 2

(Fluorescence with rhodamine red)

	Coleus	Ricinus	Geranium
Epidermis	1.4/41K1	1.9/33B6	1.3/1L9
Cork	1.9/41H9	Not observed	1.8/9L10
Cortex	0.7/3L6	1.2/41K8	1.3/1L8
Xylem	1.6/1G10	1.4/41J4	1.9/1L8
Pith	1.0/1L6	1.9/9A9	1.2/1L9

Table 3

(Fluorescence with titian yellow)

	Coleus	Ricinus
Epidermis	1.3/33B6	Too weak to observe
Cork	1.6/41B8	1.4/33E6
Cortex	1.8/1K6	1.5/41D10
Xylem	1.5/25G6	1.7/33H7
Pith	1.8/33B6	1.4/41C4

The tables show that the quality of the fluorescence varies not with the plant so much as with the tissue and with the stain used. Not only do the tables list the characteristic colors encountered in this investigation, but they also demonstrate the reliability of acridine orange. For any given tissue, table 1 shows much less variation from plant to plant than do either tables 2 or 3. The tables do not show that undifferientiated parenchymal cells have the most erratic staining properties. Of the five tissues examined, cork and xylem are outstanding in their constant reaction to the stains employed. Rhodamine red gives an even greater differentiation of the xylem areas than acridine orange, but does not stain as evenly. These stains are very sensitive and have enabled the author to detect lignification rather close to meristematic regions. Titian yellow was added for comparative purposes and for practice since the blues and yel-

lows are most difficult to compare with the standard. Its staining is not as consistent as that of acridine orange nor is it a differential stain of any importance since there is little color difference in the fluorescence of the various tissues stained with it. However if a high degree of definition is desired, titian yellow is the best of the three stains tested.

Discussion

The tables have been presented as an example of the method whereby subsequent articles on phytofluorescence by the same author shall be presented. The intensity factor is considered by the author to be the more important of the two factors comprising the complete color index. It can be determined with more accuracy than can the color comparison and it serves as a guide in the photography of the fluorescence. Furthermore Maerz and Paul state that prolonged observation of a particular color can cause retinal fatigue. This could be a common source of error in making observations on fluorescence. In addition the human eye behaves differently to very low levels of light intensity than it does to higher intensities, especially with respect to its ability to perceive color. This aggravates the problem of analyzing color since the levels of intensity of the fluorescence are quite low.

Even though sources of error do exist in the method, the method does enable different people to compare their color concepts more closely than has been the case so far. Nevertheless the basic principles of the method are sound. If a 9 -square area on the appropriate chart of Maerz and Paul be marked off and if the color index in question be the center of that area, usually the error in judgment will be within the boundaries of that area. Thus the method does enable one to say how red or how orange or how yellow a particular tissue fluoresces with more precision than has heretofore been possible.

The data can be used to compare the effectiveness of the three stains. Reference to table 1 will show the reliability of acridine orange as fluorochrome. The author has always found the same characteristic orange in the xylem parenchyma and the same characteristic yellow in the spiral thicknings of the vessels in the xylem. It is as consistent as it appears in table 1. It has enabled him to identify traces of lignin which have been subsequently proven with phloroglucinol. So constant and distinctive is this color and so characteristic only of lignified elements that acridine orange is currently being employed by the author as a differential stain for lignin.

Summary

A circular density wedge, augmented when necessary by a photographic step tablet, was employed to measure the intensity of the

fluorescent image of five tissues in *Coleus, Ricinus*, and geranium. The intensity value thus obtained was defined as the minimum density necessary to extinguish the image when the wedge was rotated through the eyepoint of the microscope. The color of the fluorescence was compared to the color standard of Maerz and Paul and the two evaluations were then listed as a fraction. The nature of the fluorescence varies more with the tissue and stain than with the kind of plant. Acridine orange was the best stain tested. It is now being used as a differential stain for lignin.

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