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## New polychrome methods in microtechnique

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NEW POLYCHROME METHODS IN MICROTÉCHNIQUE

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A Thesis

Presented to

The Faculty of the Department of Zoology

College of the Pacific

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In Partial Fulfillment

of the Requirements for the Degree

Master of Arts

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by

Celestino Nera Macabales

June 1957

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## NEW POLYCHROME METHODS IN MICROTECHNIQUE

### INTRODUCTION

When the writer took an introductory course in Microtechnique in 1954-55, he was impressed by the inadequacy of some microtechnical procedures recommended in triple staining, and he felt disappointed and discouraged by the consistently inferior results. He resolved to attempt modifications or to develop a new technique which could be relied upon to achieve anticipated results. This is a report on two years of efforts to develop a simple dependable technique in polychrome staining for histological studies. The writer feels that the results are of importance to students and teachers of biological science and to microtomists. The herein proposed technique enables the observer readily to recognize fine details of microscopic structures, and it presents a more attractive picture than does the common Harris' hematoxylin counterstained with eosin. This report also includes a list of polychrome procedures, divided into four groups: (1) hematoxylin and combinations, (2) safranin and combinations, (3) acid fuchsin and combinations, and (4) miscellaneous.

## HISTORY OF POLYCHROME STAINING

Waldayer (1863) introduced the important histological dye called hematoxylin. It is extracted from logwood. Boehmer (1865) was the first to apply hematoxylin successfully in combination with alum to obtain a fine, deep, blue, nuclear stain. Most of the subsequent development in hematoxylin staining is based upon this discovery. Today hematoxylin is a popular stain widely used both for cytological and histological staining, and it is an ingredient in some polychrome techniques.

Schwartz (1867) was the first to apply double staining by using picric acid and carmine to differentiate structures in contrasting colors. Flemming (1891) first introduced triple staining for cytological studies, using safranin as a red nuclear stain, gentian violet for mitotic spindles, and orange G for cytoplasm. Mallory (1900) introduced a triple stain for histological studies, using acid fuchsin as nuclear stain, and orange G and anilin blue, in conjunction with phosphomolybdic acid as cytoplasmic stains. These two triple stains are recommended for cytological and histological studies, respectively, by many authors; some botanists and biologists underrate their value, and others overrate them. Both these triple stains utilize orange G which has not yielded constant results in the hands of the writer, and during several years has been consistently unpredictable in

the hands of microtechnique students at the College of the Pacific. The search for a reliable substitute for this yellow stains has been a major feature of the writer's investigation.

In all the following formulas, quantities of liquids are represented in milliliters. Quantities of solids are specifically indicated.

#### Hematoxylin Combinations

##### Brillmeyer's (1929) Acid Fuchsin-Anilin Blue-Orange G

Reagents required: A. 0.2% acid fuchsin 100; B. water 100, phosphomolybdic acid 1 gram, anilin blue 0.5 gram, orange G 2 grams. Methods: The sections were first stained in Delafield's hematoxylin-water 70, ammonium alum 3 grams, hematoxylin 0.6 gram, absolute alcohol 4, glycerol 15, methanol 15. The sections were then stained in A, 1 minute and drained. They were stained in B, 2-3 hours, rinsed quickly in distilled water, then mounted in balsam via usual reagents.

##### van Gieson's (1896) Picric Acid-Acid Fuchsin

Reagents required: A. Harris' hematoxylin; B. saturated solution of picric acid 100, acid fuchsin 0.05 gram.

Methods: The sections were stained in A, 25 minutes to 1 hour, washed in water, and differentiated in acid water.

They were then stained in B, 2-10 minutes, rinsed in water quickly, and mounted in balsam via usual reagents.

Ladewig's (1938) Methyl Blue-Orange G-Acid Fuchsin

Reagents required: A. Weigert's (1903) stain-solution I, 0.4% ferric chloride; solution II, 1% hematoxylin in 95% ethyl alcohol; solutions I and II were mixed 50-50. B. 1% phosphotungstic acid; C. water 100, methyl blue 0.5 gram, orange G 2 grams, oxalic acid 2 grams, acid fuchsin 1 gram.

Methods: The tissue was fixed in formaldehyde solution. Sections were stained in A, 3-5 minutes, washed in water, mordanted in B, 2 minutes, and washed in water. They were stained in C, 4 minutes, quickly rinsed in distilled water, and mounted in balsam via usual reagents.

Lillie's (1948) Fast Green-Bismarck Brown Y

Reagents required: A. Weigert's (1904) stain-solution I, water 95, ferric chloride 0.6 gram, hydrochloric acid 0.75; solution II, 1% hematoxylin in 95% ethyl alcohol; solutions I and II were mixed 50-50; B. 0.02% fast green FCF; C. 1% acetic acid; D. 0.1% Bismarck brown Y in 1% acetic acid.

Methods: From distilled water the sections were stained in A, 6 minutes, washed in water, and differentiated in 1% acetic acid. The sections were then stained in B, 3 minutes and differentiated in C. They were stained in D, 4-6



minutes, washed in water, differentiated in C, and mounted in balsam via usual reagents. Results: Nuclei are black, general cytoplasm gray-green, mucus, cartilage, and cell granules brown.

Masson's (1912) Acid Fuchsin-Anilin Blue

Reagents required: A. 4% ferric alum at 50°C.; B. 1% hematoxylin at 50°C.; C. 2% ferric alum; D. 0.1% acid fuchsin; E. 1% phosphomolybdic acid; F. 1% anilin blue 50, 1% phosphomolybdic acid 50. Methods: Sections were taken from distilled water, mordanted in A, 5 minutes, rinsed quickly in water, stained in B, 10-15 minutes. The sections were differentiated in C, until nuclei alone were stained, washed in running water for 15 minutes, rinsed in distilled water for a few seconds, and stained in D, 10 minutes. They were then rinsed in water, mordanted in E, 5-10 minutes, stained in F, 20 minutes to 1 hour, rinsed quickly in water, dehydrated in 95% and absolute alcohol, and mounted in balsam via xylene.

Mollendorf's (1946) Eosin-Methyl Blue

Reagents required: A. Hansen's (1905) stain-water 100, ferric alum 4.5 grams, hematoxylin 0.75 gram. Alum was dissolved in 65 water and dye in 35 water. They were mixed together, boiled, cooled and filtered. B. 1% eosin in 0.3%

acetic acid; C. 2% phosphomolybdic acid; D. 1% methyl blue.

Methods: Sections were taken from distilled water, stained in A, 5 minutes, rinsed in water and stained in B, 20 minutes. The sections were washed in water, mordanted in C, 10 minutes, rinsed in water. They were stained in D, 1-2 minutes, rinsed quickly in 95% alcohol, mounted in balsam via usual reagents.

Paquin and Goddard's (1947) Picro-Phloxine-Orange G-Anilin Blue

Reagents required: A. water 75, 95% alcohol 25, glycerol 13, ferric alum 5 grams, ammonium sulfate 0.7 gram, hematoxylin 0.8 gram. Preparation: The dye was dissolved in the glycerol and alcohol with gentle heat; the other ingredients were dissolved in water and mixed with the dye with constant agitation and left for 24 hours. B. 0.5% picric acid in 95% alcohol; C. water 100, phosphotungstic acid 0.1 gram, eosin 0.07 gram, phloxine 0.03 gram, orange G 0.1 gram; D. 2% phosphotungstic acid; E. 0.4% acetic acid; F. 0.04% anilin blue in 1% acetic acid. Methods: The tissues were fixed in Masson's (1947) fixative-water 187.5, picric acid 2.2 grams, chrome alum 7.5 grams, 40% formaldehyde 67.5; the alum was soaked in the formaldehyde for 1 hour before adding the picric acid that was dissolved in water. It was left for 24 hours and filtered. The sections were transferred from water to A, 5 minutes, washed in running water for 5 minutes, stained in B, 15-20 seconds and rinsed in water; the sections were stained in C, 5 minutes, mordanted in D, 5 minutes and

differentiated in E, dipped twice. They were then stained in F, 5 minutes, differentiated in E, 30 minutes. The sections were dehydrated in 95% and absolute isoamyl alcohol, cleared in toluene and mounted in balsam. Results: nuclei black, elastic tissues cherry red, other connective tissues blue, general cytoplasm pink.

Patay's (1934) Ponceau 2R-Light Green

Reagents required: A. Masson's (1934) stain-water 100, acetic acid 2, hematin 2 grams, potassium alum 6 grams; the alum was dissolved in boiling water, and the dye was added. The mixture was cooled and filtered, and the acid was added to the filtrate. B. 1% ponceau 2R; C. 1% phosphomolybdic acid; D. 0.5% light green in 90% alcohol. Methods: The sections were transferred from distilled water to A, 30 minutes to 1 hour, washed in water, differentiated in acid water. They were stained in B, 2 minutes, rinsed in water, mordanted in C, 2 minutes, briefly rinsed in water, stained in D, 30 seconds, and mounted in balsam via usual reagents. Results: nuclei and cartilage blue, other collagens light green, bones brilliant green, epithelial tissues and muscles orange, erythrocytes yellow, nervous tissues gray.

Reeve's (1948) Safranin-Fast Green

Reagents required: A. water 90, Delafield's (1885) hematoxylin 10-water 70, ammonium alum 3 grams, hematoxylin 0.6

gram, absolute alcohol 4, glycerol 15, methanol 15; B. water 40, 95% alcohol 60, safranin 0.01 gram, sodium acetate 0.01 gram; C. xylene 75, absolute alcohol 25, saturated with fast green FCF in 50:50 clove oil. Methods: The sections were transferred from distilled water to A, 5-15 minutes, washed and differentiated in acid water. They were stained in B, 5-15 minutes, rinsed in 95% alcohol for a few seconds, stained in C, 1-3 minutes, mounted in balsam via usual reagents.

#### Safranin Combinations

##### Conant's (1940) Crystal Violet-Fast Green-Orange II

Reagents required: A. 1% safranin in 50% alcohol; B. 1% crystal violet; C. 1% fast green in absolute alcohol; D. saturated solution of orange II in clove oil. Methods: The tissue was fixed in formalin-acetic-alcohol. The sections were transferred from 70% alcohol to A, 2-24 hours, rinsed in water, differentiated in acid water and stained in B, 1 minute. They were rinsed in water, dehydrated in series of alcohol, stained in C, few seconds, stained in D for 2 changed, mounted in balsam via xylene.

##### Henneguy's (1898) Methyl Violet-Orange G

Reagents required: A. water 100, ammonium thiocyanate 1, methyl violet 0.1 gram, orange G 0.1 gram; B. Zwaardemaker's

(1887) stain-95% alcohol 50, safranin 1.5 grams, saturated aqueous solution of aniline 50. Preparation: The dyes were dissolved in alcohol, and aniline was added. Methods: The tissue was fixed in osmic-chrome-acetic fixative. The sections were transferred from water to A, 10 minutes, rinsed in water, stained in B, 15 minutes, dehydrated in absolute alcohol for a few seconds, mounted in balsam via xylene and clove oil.

Johansen's (1940) Methyl Violet-Fast Green-Orange G

Reagents required: A. 95% alcohol 25, methyl cellosolve 50, safranin 0.1 gram, sodium acetate 1 gram, 40% formaldehyde 2. Preparation: The dye was dissolved in cellosolve, and the alcohol was added then the water, and the remaining ingredients; B. 1% methyl violet; C. 95% alcohol 30, ethylene glycol monomethyl ether 30, tertiary butyl alcohol 30; D. clove oil 6, ethylene glycol monomethyl ether 6, fast green enough to saturate 95% alcohol 36, tertiary butyl alcohol 50, acetic acid 0.5; F. saturated solution of orange G in ethylene glycol monomethyl ether 30, 95% alcohol 30; G. clove oil 30, ethylene glycol monomethyl ether 30, 95% alcohol 30; H. clove oil 30, absolute alcohol 30, xylene 30. Methods: The sections were transferred from 70% alcohol to A, 1-2 days, rinsed in water, stained in B, 10-15 minutes, washed in water, rinsed in C, 15 seconds. They were stained in D, 10-15 minutes, rinsed briefly in E, stained in F, 3 minutes,

differentiated in G and H, mounted in balsam via xylene.

Results: dividing chromatin red, resting nuclei are purple, lignified and suberized tissues red, cellulose green-orange, cytoplasm bright orange, starch grains purple, fungal mycelia green. These methods are recommended for plant tissues.

Stockwell's (1934) Gentian Violet-Orange G-Picric Acid

Reagents required: A. 1% chromic acid; B. 1% crystal violet 20, 1% safranin O 40, water 40; C. LaCour's (1931) stain-80% alcohol 100, potassium iodide 1 gram, iodine 1 gram; D. 1% picric acid in 95% alcohol; E. 95% alcohol 100, ammonium hydroxide 0.3; F. 0.2% orange G in clove oil. Methods: The sections were transferred from distilled water to A, 1 hour to overnight, washed thoroughly in water, stained in B, 1-6 hours, rinsed in water, stained in C, 30 seconds. They were then rinsed quickly in 70% alcohol, stained in D, few seconds, differentiated in E, few seconds, mounted in balsam via usual reagents.

Acid Fuchsin and Combinations

Bensley's (1938) Orange G-Anilin Blue

Reagents required: A. Altmann's (1890) stain-saturated solution of anilin 100, acid fuchsin 20 grams; B. 1% phosphomolybdic acid; C. water 100, orange G 2 grams, anilin blue 0.5 gram. Methods: The sections were taken from distilled

water to A, 10 minutes, rinsed quickly in water. They were stained in C, 1 hour, mounted in balsam via usual reagents.

Bohm and Oppel's (1907) Orange G-Brilliant Cresyl Blue

Reagents required: A. Solution I, water 100, orange G 0.05 gram, acid fuchsin 0.05 gram, acetic acid 1, 40% formaldehyde 1; solution II, water 90, methanol 10, 0.25% brilliant cresyl blue 0.25, 40% formaldehyde 1; solutions I and II 50+50. Methods: The sections were taken from water to A, 20-30 minutes, differentiated in absolute alcohol, mounted in balsam via xylene. Results: nuclei red-purple, cartilage blue, bones orange, muscles red.

Kricheski's (1931) Methyl Blue-Orange G

Reagents required: A. 0.25% acid fuchsin; B. 2% methyl blue 30, 1% orange G 30, 1% phosphomolybdic acid 30. Methods: The sections were taken from distilled water to A, 1-3 minutes, rinsed in water, stained in B, 3-5 minutes, rinsed in 70% alcohol, mounted in balsam via usual reagents. Results: nuclei red, collagen blue, nerves and glands violet, muscle red, erythrocytes and keratin orange.

Milligan's (1946) Orange G-Fast Green

Reagents required: A. water 75, 95% alcohol 25, potassium dichromate 2.25 grams, hydrochloric acid 2.5; B. 0.1% acid

fuchsin; C. 1% phosphomolybdic acid; D. 2% orange G in 1% phosphomolybdic acid; E. 1% acid; F. 0.1% fast green in 2% acetic acid. Methods: The sections were mordanted in A, 5 minutes, rinsed in water, stained in B, 5 minutes, and rinsed in water. They were mordanted in C, 1-5 minutes, stained in D, 5-10 minutes, differentiated in E, 2 minutes, stained in F, 5-10 minutes, differentiated in E, 3 minutes, rinsed in 95% alcohol, and mounted in balsam, via usual reagents.

Note: The tissue was fixed in formaldehyde mixture.

#### Miscellaneous Combinations

##### Buzaglo's (1934) Gallocyanin-Orcein-Acid Alizarin Blue-Alizarin Viridine

Reagents required: A. Becher's (1921b) stain-water 100, chrome alum 5 grams, gallocyanin 0.5 grams. Preparation: The mixture was boiled, cooled, and filtered; B. 70% alcohol, hydrochloric acid 1, orcein 1 gram; C. water 100, aluminum sulfate 10 grams, acid alizarin blue 5 grams; D. 5% phosphomolybdic acid; E. water 100, hydrochloric acid enough to make pH 5.8, alizarin viridine 0.2 gram. Preparation of C: The mixture was boiled 10 minutes, cooled and filtered. Methods: The sections were taken from water to A, 24 hours, rinsed in water, stained in B, 3 changes 5 minutes each, rinsed in water, stained in C, 7 minutes, rinsed in water, and mordanted in D, 1-5 minutes; they were



rinsed in water, differentiated in E, 7 minutes, dehydrated in absolute alcohol quickly, and mounted in balsam, via carbol-xylene and pure xylene. Results: nuclei dark blue, elastic fibers brown, muscles blue, cartilage green.

Castroviejo's (1932) Magenta-Picro-Indigocarmine

Reagents required: A. water 100, 40% formaldehyde 0.6, Ziehl's (1882) stain 5-water 100, 90% alcohol 10, magenta 1 gram, phenol 5 grams. Preparation: The dye was ground with the phenol in a mortar; when they were dissolved the alcohol was added in 10 successive lots while grinding, then the mixture was filtered; B. acetic acid 0.6, Cajal's (1895) stain 100-water 100, picric acid 1, indigocarmine 0.25 gram. Methods: The sections were taken from distilled water to A, until nuclei well stained, washed in water, stained in B, until cytoplasm well stained, rinsed in water, and mounted in balsam via usual reagents.

Kornhauser's (1943) Orcein-Acid Alizarin Blue-Orange G-Fast  
Green

Reagents required: A. 95% alcohol 90, water 10, nitric acid 0.4, orcein 0.4 gram; B. water 100, crystal aluminum sulfate 10 grams, ferric chloride 0.8 gram, acid alizarin blue 2 B 0.35 gram; C. 5% phosphotungstic acid; D. water 100, acetic acid 2, orange G 2 grams, fast green FCF 0.2 gram.

Preparation: The dyes were boiled in sulfate solution 10 minutes, and ferric chloride was added in a little water.

Methods: The sections were taken from 85% alcohol, to A, 2-24 hours, rinsed in 85% alcohol, washed thoroughly in water, via graded series of alcohol, stained in B, 5-10 minutes, and mordanted in C, 10-30 minutes. They were rinsed quickly in water, stained in D, 10 minutes, rinsed in 50% alcohol, and mounted in balsam via usual reagents. Results: nuclei red, elastic fibers brown, cytoplasm and muscles violet, collagen green, erythrocytes orange. Note: Tissue was fixed in mercuric-chromic mixture.

Kull's (1913) Toluidine Blue-Aurantia-acid Fuchsin

Reagents required: A. saturated solution of aniline 100, acid fuchsin 20 grams; B. 0.5% toluidine blue; C. 0.5% aurantia in 70% alcohol. Methods: The sections were taken from distilled water, warmed in A to steaming, 1 minute, cooled, rinsed quickly in water, stained in B, 1-2 minutes, and rinsed quickly in water. They were stained in C, 20-30 seconds, differentiated in 95% alcohol, and mounted in balsam via usual reagents. Note: This procedure was originally intended for demonstration of mitochondria, but they were also excellent for general-purpose stain.

Margolena's (1933) Phloxine-Orange G

Reagents required: A. 0.5% phloxine in 20% alcohol; B. 0.5% orange G in 95% alcohol. Methods: The sections were taken from distilled water, stained in Harris' Hematoxylin or celestin blue, rinsed in water, stained in A, 1-5 minutes, rinsed in 70% alcohol, and stained in B, by dropping the red dye on the slide, 30 seconds to 1 minute. They were dehydrated in absolute alcohol, and mounted in balsam via toluene.

Matsura's (1925) Polychrome Neutral Red

Reagents required: A. 1% congo red in 95% alcohol; B. 1% phosphomolybdic acid in absolute alcohol. Methods: The sections were taken from distilled water, to A, 12-24 hours, rinsed in absolute alcohol, differentiated in B, 5 minutes, dehydrated in absolute alcohol, and mounted in neutral mountant via oil of thyrne. Results: nuclei red, elastic fibers red-violet, collagen green, white blood cells violet, other tissues brown.

Shumway's (1926) Magenta-Picro-Indigocarmine

Reagents required: A. 1% magenta; B. 0.2% indigocarmine 50, 1.2% picric acid 50. Methods: The sections were taken from distilled water to A, 20 minutes, stained in B, 5 minutes, rinsed in 70% alcohol until pink, few seconds, rinsed in absolute alcohol till blue-green, and mounted in balsam via

usual reagents. Results: resting nuclei dark blue, mitotic figures dark red, cartilage pink, procartilage light blue, bones dark blue, muscles bright green, nerves purple.

Williams' (1935) Cresyl Violet

Reagents required: Cresyl violet 1 gram, anhydrous potassium carbonate 1 gram, water 95, 40% formaldehyde 5. Preparation: The dye and potassium carbonate were dissolved in water and formaldehyde; the mixture was shaken for 30 minutes, 3 acetic acid was added slowly with constant agitation. The mixture was shaken for 30 minutes, filtered, and 5 absolute isopropyl alcohol was added. Methods: The tissue was sectioned in freezing microtome without fixing. The sections were taken from distilled water to stain, 6 seconds, washed and examined under the microscope. Results: nuclei and muscles blue, fat yellow, other tissues pink. Note: This is recommended for rapid clinical diagnosis.

## RECOMMENDED PRESTAINING PROCEDURES

Prestaining procedures consist of standard methods of killing, fixing, washing, dehydrating, clearing, infiltrating, imbedding, sectioning, stretching, and affixing sections to the slides. The following special recommendations are based upon the writer's experience with frog tissues. The stomach and the tongue were selected for most comparisons of results.

### Killing and Fixing

Prior to killing, the frog was starved for three days to empty its digestive tract. In order to avoid chemical variables accompanying the use of lethal drugs, the writer dissected the frog while it was still alive. The tissues were washed with physiological saline, divided into small pieces, and fixed in Zenker's fluid (which proved to be superior to other fixatives used) for 24 hours.

### Washing

The tissues were washed in running tap water for 24 hours after fixation, passed through a graded series of 30%, 50%, 70% isopropyl alcohol for one hour in each. Removal of residual traces of mercuric chloride was not attempted until after sectioning.

### Dehydrating

As recommended by Gray (1954) the specimens were suspended toward the top of a vial of alcohol in order that the water extracted from them might diffuse toward the bottom of the vial. The tissues were covered with 50 times their volume of isopropyl alcohol, and the vial was tightly covered at all times except when changing the alcohol. The 70% alcohol was changed to 85%, 95% alcohol one hour each, and absolute isopropyl alcohol twice for six hour periods.

### Clearing

The absolute isopropyl alcohol was changed with one-third toluene and two-thirds absolute isopropyl alcohol for 15 minutes, one-half toluene and one-half absolute isopropyl alcohol for 15 minutes. Two changes of pure toluene were made for one hour each.

### Infiltrating

The toluene was changed with one-half toluene one-half melted paraffin inside the paraffin oven at 55°C for three hours. Two changes of melted paraffin were made for six hours each.

### Imbedding

The bottom of a syracuse watch glass was coated with glycerine by rubbing with a moistened finger. The melted paraffin containing the tissues was poured rapidly into the syracuse watch glass, and the tissues were oriented with a clean, warm needle. When the paraffin had congealed sufficiently and formed a thin surface film, it was cooled by quickly plunging the watch glass into cold water. The paraffin block rose to the surface as soon as it had thoroughly cooled.

### Blocking and Trimming

The block was roughly trimmed, attached to the object carrier by means of a warm spatula. The block was later trimmed carefully with a razor blade till a plane figure of four sides was produced, two of which were parallel.

### Sectioning

The object carrier was inserted in the jaws of a rotary microtome. The thickness was set to 10 microns, and the handle of the microtome was rotated. A ribbon tray received the long ribbon as it was sectioned.

### Stretching and Affixing Sections to the Slides

The ribbon was divided into segments of two or three sections each. A clean slide was coated with a thin film of Mayer's albumen fixative. The surface of the slide was flooded with boiled, distilled water, and the sections were floated and warmed gently over an alcohol lamp until the paraffin flattened without being melted. The sections were moved to the center of the slide with a needle and the water was drained. The slide, protected from dust, was dried overnight.



## TWO NEW POLYCHROME PROCEDURES

### Basic Fuchsin-Anilin Blue-Picric Acid

#### Reagents Required

After experimenting with various strength of dyes, the following formulas proved to be satisfactory: Basic fuchsin 0.5% in 85% acetone, anilin blue 0.5% aqueous solution, picric acid 0.0625% in toluene (0.125 gram in 200 toluene).

#### Methods

The sections affixed to the slide were taken from distilled water, washed in Lugol's solution for 5 minutes, rinsed in 5% sodium thiosulfate for a few seconds, and briefly washed in water. The sections were stained in anilin blue for 1 minute, rinsed in 85% acetone for a few seconds, stained in basic fuchsin for 2 minutes, rinsed in 85% acetone, and then washed in pure acetone for a few seconds. The sections were stained in picric acid for a few seconds, rinsed in pure acetone, and then immersed in 85% acetone (in water) for differentiation of the anilin blue. Dehydration was then accomplished in pure acetone, clearing in toluene. If the microscope indicated insufficient differentiation, the slide was agitated successively in 85% and pure acetone for a few seconds and then quickly dipped in

picric acid. The sections were washed in toluene, covered with a few drops of clove oil for a few seconds, cleared in three changes of fresh toluene, and mounted in balsam or other resinous media. The use of clove oil made more delicate differentiation possible and imparted greater durability of the dyes as demonstrated by exposures to sun and heat.

### Results

The nuclei were stained red, connective tissues light and dark blue, muscular tissues light and dark green; other constituents stained yellow, orange, pink, and purple.

### Celestin Blue B-Phloxine-Yellow Food Color

#### Reagents Required

Celestin blue B 0.5 gram, ferric alum 5 grams, water 100, mixed, boiled for 5 minutes, cooled and filtered; 1% phloxine in aqueous solution; yellow food color 3 in 85% ethyl alcohol 50.

#### Methods

The sections affixed to the slide were taken from distilled water, washed in Lugol's solution for 5 minutes, rinsed in 5% sodium thiosulfate for a few seconds and briefly

washed in water. The sections were stained in Celestin Blue B for 10-30 minutes, rinsed in water for few seconds, stained in phloxine for 5-15 minutes, rinsed in two changes of 95% alcohol and stained in yellow food color for 1-10 minutes. They were observed while staining and further differentiated in 85% alcohol. They were then rinsed in fresh 85% alcohol quickly and mounted in balsam via usual reagents.

### Results

The nuclei were stained blue, muscular tissues red, connective tissues yellow and orange. Various colors played minor roles.

## DISCUSSION

These two new polychrome procedures, Basic Fuchsin-Anilin Blue-Picric Acid and Celestin Blue B-Phloxine-Yellow Food Color, differ in miscibility of the solvent used. Picric acid is the only stain miscible with toluene. Both these polychrome procedures are simple and have yielded results superior to others used in the past. Most of the hematoxylin, miscellaneous combinations and all the safranin, acid fuchsin combinations in the list utilized orange G which had yielded unreliable results in the hands of the writer. He substituted yellow food color for orange G with satisfactory results.

Yellow food colors are manufactured for food coloring purposes by McCormick & Company, Inc., Schilling Division, San Francisco, California. They are a blend of (a) food, drug & cosmetic Yellow # 5 (b) food, drug & cosmetic Red # 2. The Yellow # 5 is in the preponderance, commonly known as Tartrazine, having a color index number of 640, and classified as Pyrazolone. Red # 2 is used primarily to shade-off the natural color of the yellow, commonly known as Amaranth, having a color index number of 184, and classified as Monoazo. The solvent of the yellow food color is a water solution containing sufficient propylene glycol to inhibit fungus and bacterial growth. They are standardized and

certified by the company. Mr. C. V. Finell, Chief Chemist of the Company, kindly supplied the writer with this information although it was not possible for him to reveal the chemical formulae of their products.

While the discovery and use of one primary color (yellow) as a reliable substitute for the traditional orange G is a source of satisfaction to the writer, the possibilities of further explorations along these lines are, indeed, intriguing. His efforts will be continued.

## SUMMARY

Two new polychrome methods in microtechnique, Basic Fuchsin-Anilin Blue-Picric Acid and Celestin Blue B-Phloxine-Yellow Food Color, have been developed. So far as histological staining is concerned, these, in the writer's opinion, are superior to the methods which have been vague for many years. Most polychrome procedures of the past which utilized orange G gave variable and unpredictable results. The writer found that Schilling yellow food color was surprisingly satisfactory as a microtechnical stain, and he was able to incorporate it in combination of Celestin Blue and Phloxine.

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