Multispectral techniques applied to photomicrography

William Richard Mueller

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Multispectral Techniques Applied to Photomicrography

by

William Richard Mueller

A thesis submitted in partial fulfillment of the requirements for the degree of Bachelor of Science in the School of Photography in the College of Graphic Arts and Photography at Rochester Institute of Technology

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Thesis Advisor; Dr. G. W. Schumann
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ABSTRACT

Based upon an object's absorption and reflection characteristics to varying amounts of radiation, a series of selective wavelength band exposures were made for color separation of a microscopic specimen. The exposures are made to record the specimen's characteristic transmission or reflection patterns as a visible density value. The exposures were made in an overlapping band width manner. The exposures were in the blue, green, and red regions of the spectrum. The resultant negatives were printed on Polycontrast paper using filter No. 2.

The main purpose of the experiment was the design of a photographic system to remove the biological step of staining. This was through the use of the spectral transmission curves of the component parts of the specimen. The results of the separations must then be referenced to the spectral output of the specimen's component parts. This was not tried because of the region of the spectrum that was needed was in the far ultraviolet.
INTRODUCTION

Upon several discussions with fellow students in the field of biology, our talks generated around a problem caused from staining techniques needed in viewing a microscopic specimen. The author was presented with several problems that occurred in staining a specimen for microscopic viewing. Staining techniques caused several problems in the recognition of a specimen's cellular structure. In biological fields there are many specimens that need a stain to visually them microscopically. These stains are also needed to determine the specimen's cellular anatomy through microscopic viewing.

Effective staining is a difficult technical procedure involving several factors and steps. Two factors involved in specimen staining are under and excess stain, and the hues used in staining. Understaining a specimen can cause problems related to saturation of the stain for a needed visual density difference, or needed hue contrast between parts of the specimen's anatomy. Excess staining causes a increase in visual density to a point where specimen details can be obscured by the presence of the stain. Excess stain decreases the contrast differences through a higher overall density. The problems of under and excess staining cause a unwanted variable in subject identification of microscopic specimens. Specimen staining problems arise in visual determination of a specimen's identity. In photographic reproduction stain can cause unwanted color shifts through normal color reproduction methods.
To improve a stained specimen's anatomy identification a photographic system of multiple radiation band exposures could be employed. The photographic system would be able to control hue, saturation, and image contrast through an additive viewing process. The recorded black and white separation images would give control in the selection of image records to be viewed. The additive viewing system can control hue changes and saturation changes within the hues. This would create a maximum in contrast and color control during final viewing observations.

A three color additive system may not suffice if limited to the visible spectrum. Therefore an extension into the infrared radiation region should be employed. With the extended exposures into the infrared there is a possibility of a infrared false color representation of the specimen. The infrared radiation region will permit a extended multispectral system of exposure records to increase information about the specimens absorption characteristics in visual and infrared radiation regions. This is corresponding to the wavelength bands selected for the separation records.

Multispectral Photographic Background

A literature search has found applications of multispectral photography in the field of aerial photogrammetry. Familiar problems in aerial photogrammetry use the multispectral system for image enhancement of the photographed subject. Multispectral photography has been applied to the fields of geomorphology (the study of the characteristics, origins, and development of land forms), mineralogical analysis
of a terrain (a study of mineral content on the surface of the land area), coastal and water studies (depth penetration, interfaces between fresh and salt water bodies, pollution control, and sedimentary deposits in river regions), forestry and crop infestation and specifi-
cation. Other problems are in aerial topography, mapping and aerial reconnaissance. The majority of problems are related to a ground objects spectral reflectance characteristics. The multispectral photographic system depends upon an objects characteristic absorption or reflection to varying radiation wavelengths. The systems multiple wavelength band exposures can find abnormalities in soil and surrounding vegetation by deviations from a normal characteristic reflectivity. Usage of the infrared radiation region affords false color investigation in camouflage detection and crop disease detection.

The aerial photogrammetric application can be explained in the following manner. The multispectral system consists of breaking down the visual spectrum into its three major components of red, green, blue, and extending the spectral range into the infrared. Four black and white separation images are exposed and processed so that their gammas are approximately equal. A positive duplication is made from the separation negatives with a low to medium contrast. The positive images are recombined through additive filtration to make a positive image record. A normal color rendition of the scene is made from the red, green, and blue spectral images. After a reference is made of the normal color rendition, the systems false color abilities allow the recorded images to act as separation masks to one another. The ability to change the response hue and saturation of each recorded
image giving controlled false color representation of the original object. Changing the hue and saturation promotes an increase in hue contrast and tonal differentiation for maximum definition in subject areas.

The premise for multispectral photography is that all objects have a characteristic spectral response. Objects when exposed to varying incident radiation flux exhibit a characteristic absorption or reflection response. These characteristic patterns from an object are its response variable to varying radiation flux. This variable can be recorded photographically as densities. The densities recorded are the objects' response output in the selected exposure bands.

In the aerial photogrammetric application of multispectral photography the wavelength bands under investigation are from the near ultraviolet at approximately 325nm through the visual spectrum extending into the infrared to approximately 900nm. The infrared limits are in occurrence with spectral senitization of films to infrared radiation. Additional exposures from the infrared region allow extended false color representation with applied cross filtration techniques capable through the viewing system.

The regions under investigation are blue from 325nm to 520nm, green from 480nm to 620 nm, red from 590nm to 710nm, and infrared from 700nm to 900nm. The separation of the visual spectrum into its component parts of red, green and blue give the normal additive color separation for the object scene. The exposure extension into the infrared region gives an added investigation of an object's characteristics to the longer radiation wavelengths.
The choice of filters were made to cover completely the visible spectrum, also to approximate the color sensitivity of the standard observer in the human eye. This filter selection will permit comparison of the multispectral system with any of the conventional color or color infrared reproduction systems. After the initial set of exposures are made the spectral separation negatives are processed so that the density of any image image on each individual spectral negative is a correct representation of the brightness for that object. A gamma is chosen to produce a medium contrast without excessively reducing the exposure range. Positive transparencies are made with a medium contrast and a low minimum density.

It can be stated that the viewer has the ability to select either a normal color representation of the subject or a false color representation. Through the viewing system the positive images are placed in the same reference plain with respect to the taking position. The viewer has four individual projection systems with each projecting a separation image. The four individual projection system allow filtration and intensity changes of each image without affecting the other images hues and saturation being projected. The four individual projected images when combined have a individual effect on the final projected image. The observer can select singular or combinations of the four projectable images with any hue or saturation combination desired. This allows selection of spectral records that are wanted in investigating the subject scene. Based upon the spectral records wanted the observer can select any type of false color rendition, or normal rendition of the subject can be exhibited on the view screen.
Multispectral verses Standard Color Photographic Techniques

Through the literature search it is expected that the multispectral technique will afford greater freedom in final selection of image areas contrasted and separated by cross filtration and saturation control techniques. This based upon the spectral reflectance characteristics of the object recorded as density on the separation negatives. The individual exposures of red, green, blue, and infrared are different from standard methods of color reproduction using a single three layer emulsion.

Presently standard color and false color photographic techniques are in relationship to a single three layered emulsion for color and color infrared image reproduction. The present techniques in color and color infrared photography are bound by preselection of spectral sensitized regions and set dyes in the emulsion layers of the film. Two examples of standard color reproduction films are Kodak Ektachrome Infrared, with the second being Kodak Ektachrome Daylight color film.

These two films are bounded in there color response abilities. The Ektachrome Daylight responds to Red, Green, and Blue radiation flux. The three exposed layers are restricted in thier dye response with Cyan, Magenta, and Yellow always corresponding to the above exposure regions. Similiarly Ektachrome Infrared is restricted to exposures Green, Red, and Infrared radiation. These layers have corresponding dyes of Yellow, Magenta, and Cyan. The sinle three layered emulsion films do not allow the cross dyeing or exposure band width selection. This is the major drawback in the use of conventional three layered
color films used for color reproduction. Therefore the multispectral systems added demension of cross dying and exposure band width selection gives a totally controled false false color rendition of the final image.

Multispectral Photomicrography

Several of the ideas that were presented in the preceeding text lead to a possible application of multispectral photography to the field of photomicrography. The application of multispectral photography to a microscopic specimen leads to several questions.

1.) Can the usage of a multispectral system of photography on a microscopic specimen cause the characteristic biological absorption traits to be recorded.

2.) Is the system capable of recording a unstained specimen to give a clear representation of the specimens structure spectral absorption traits.

3.) Does a multispectral system give needed image enhancement of a recorded microscopic specimen.

4.) Is the system able to determine the difference between the stained and the unstained specimen due to absorption characteristics of the specimens.

The questions that were just presented can be explained for the normal color separation techniques. This leads to the application of the extension into the infrared region of the radiation spectrum. Does the extension into the infrared region promote or add to photo-
graphic reproduction of the microscopic specimen.

A literature investigation was made to find applications and tech-
iques of the infrared region to photomicrography. The infrared region
of the spectrum was applied to specimens for recording subject penetra-
tion in a microscopic specimen. In 1932 Kraft worked with lower
silurian grapteloties and found that even the carbonized chitin remains,
such as fish scales and animal hairs, lent themselves to infrared photo-
graphy.

Applicational problems occur when infrared sensitized materials
are used. Infrared photography would not be used to obtain a better
resolution, because you are forced to accept a poorer resolution when
using this region of the spectrum. The preferrable reasons for the
usage of this type of illuminanation are to obtain image contrast as
determined by the relationship between the absorption absorption bands
of the specimen and the wavelengths included in the recorded illuminanation.

Other problems involved are in the focusing of the image due to the
increased wavelength size in this area of the radiation spectrum.

Since most stains have little or no absorption in the infrared,
another application of the same general nature is its usage to reduce
the intensity of the stain of even to obtain the same apperance as if
the specimen had not been stained. Another application is that
infrared is employed to obtain enhancement of contrast when portions
of the specimens details have infrared absorption characteristics.

It is hoped that with the extension into the infrared these recorded
images will be examples of a true penetration record and specimen
response.
The experimental results will be tested against the following photographic reproduction systems, a false color, a normal color transparency, a color negative process and a positive print. These are the conventional color reproduction films and methods that the multispectral system will be tested against. It is thought that through the multispectral system a increased definition in hue, saturation, and contrast will be obtained in the final reproduced image. This system's ability will hopefully decrease the needed staining procedures in viewing of microscopic specimens.
EXPERIMENTATION

This chapter will be concerned with the methods that were used in the research experiment. There will be discussion on several problems encountered and the alternate methods that were used in the research of the experimental problem. Experimental results will be reported to the point of completion during the time period allotted for the experiment.

The requirements of the experiment are to correlate the spectral response of each of the wavelength bands to a spectrophotometric record of the specimen.12 This is to correlate the spectral absorption that the specimen has to a resultant density on separation negatives.

The film processing is critical for accurate results. The panchromatic film used may have a different characteristic curve as a function of wavelength. Therefore the characteristic curves must be checked for differing responses as a function of the radiation region exposing the film. This promotes the problems of each of the spectral bands having a different relationship of density verses log exposure.13 To correct this processing can be done through differential processing.14 The positive duplication should be made for a low base density and a moderate to high overall contrast.

Specimen Selection

At the time of the research proposal it was hoped that specimens from a carbonized change such as chitins could be used. This was to
allow maximum efficiency for infrared capabilities in subject penetration. It was proposed that fruit flies and human cheek cells would be a good representative of a carbon tissue family. Botanical specimens were also proposed as onion skin cells and anacharia (green plant cells) to allow an extension into a varied field.

Upon discussions and a research investigation it was found that the proposed specimens would not be suitable for the experimental investigation. It was felt that the specimen should be changed to an origin tissue from a general family of organs. Limitations were set upon the origin selection, first its anatomy had to be easily visible at lower magnification powers, secondly it had to be easily separable in visual structural components for visual examination.

The specimen selection was made after discussions with Dr. J. Baird Head of the biology department at R.I.T. and Dr. M. Hyman of Ward's Natural Scientific Establishment Inc. It was suggested that tissues from the rodent family be used. The two experimental specimen tissues selected were to be paraffin sections from a rat's adrenal glands and the second from a rabbit's adrenal glands. It was hoped this specimen would give both a refinement in cellular structure and a good sample of origin type tissue. The reason for the selection of the adrenal glands was the easy differentiation between the parts of the cells anatomy. That is the difference between the cytoplasm, the nuclei, and the cell's wall structure at a low magnification power.

Histological Technique

The experimental format was to hopefully test a stained specimen
slide verses a nonstained specimen slide. The following is a discussion on the techniques and the results obtained from the methods used in sectioning and staining the specimen slides.

To provide a secondary testing of the multispectral photographic system two independent parties were used for specimen slide preparations. The rabbits adrenal gland sections were prepared at Ward's Natural Scientific Establishment by a Miss Sharon Mante. The rats adrenal glands were prepared by Miss Robin Demuth a Medical Technology student at R.I.T.. Both techniques were preformed in an identical manor for testing the possibility of human error in preforming the sectioning and staining technique.

The orgins were prepared for a paraffin sectioning technique. Fixation was with a formilin solution of 10cc Commercial formilin to 90cc of distilled water. The fixation time was approximately 65 hours. A dehydration process follows to remove the water in the specimen. Dehydration was through several stages of alcohol solutions starting with a solution of 70% alcohol through the final steps of washing in absolute alcohol.

The specimen must than go through a clearing process. This is to displace the alcohol with cloroform which is mixable with paraffin. Therefore a cloroform and alcohol wash is needed with a final wash in a cloroform solution. The process starts after the dehydration process. The process is similar to the dehydration process in that you are replacing the alcohol with a cloroform solution. The process is by usage of successive solutions of a absolute alcohol and cloroform with a increase in the cloroform content through each bath. The time in each solution is twice the time that it takes the specimen to sink in the solution. Changing to a cloroform solution in the
final bath for a time period of 40 minutes. Extract from the solution and start the infiltration process.

Infiltration is the step where the embedding material is penetrated through the specimen. This is in preparation for embedding the specimen into a paraffin block. The steps proceeded as follows. The specimen was transferred from the chloroform solution through the following paraffin infiltration steps. Paraffin materials and times in the paraffin, (1) soft paraffin - melting temperature $48^\circ C \pm 1^\circ C$ for 15min. (2) medium paraffin - melting temperature $52^\circ C \pm 1^\circ C$ for 15min. (3) hard paraffin - melting temperature $56^\circ C \pm 1^\circ C$ for 15min.

The process that is after the infiltration is called embedding. This is the final step before the microtomeing of the specimen. Prepare a molding block with the dimensions of $1\frac{1}{2}\" \times 1\" \times \frac{1}{2}\"$. This is the embedding mold for the specimen. Place the mold on a level surface, using the hard paraffin fill the mold level full. Transfer the specimen from the last paraffin change into the paraffin mold. Position the specimen in the block and place in a cold water bath to harden. When in the cold water make sure that the water doesnot enter the paraffin block. When cooled the mold is removed and the specimen is ready for microtome sectioning.

The specimen is than positioned in a microtome for sectioning. The sections were made are thickness of 6u. The section ribbon was taken and placed aside for separation into a series of slides. The was made for a minum of 20 microtome slices for a progressive ordered set of slides. The reason for the 6u thickness was to achieve a singular celled structured layer.
The specimen sections were affixed to a standard slide with a album solution and allowed to dry for 48 hours. The slides were numbered 1 - 20 in preparation for the staining procedure that was used. The slides were cleared of the paraffin by reversing the clearing process used earlier. This was with successive alcohol and chloroform solutions to displace the paraffin that was needed for the microtome sectioning of the specimen.

The slides were in preparation for hydration to be stained or affixed without the staining steps. The slides were rehydrated through successive solutions of absolute alcohol down to a 70% alcohol solution. The even numbered slides were then affixed permanently with their cover slides. The R.I.T. slides were affixed with Canadian Balsam while the Ward's slides were with a Cadine affixitive. The odd numbered slides were stained using Ehrlich's Hematoxylin and certified Erosin counterstain. The slides were permanently affixed in the same manner as the even numbered slides.

Results were received from Ward's natural Scientific Establishment with the 6u sections and the hematoxylin and erasin stain. The slides were numbered 1 - 20 with the odd numbered slides being stained and the even numbered slides cleared and affixed. The slides were not completed at R.I.T. due to time limitations upon Miss Demuth. Miss Demuth supplied other slides that were used for test experimentation in trial photographic set up.

Spectral Transmission Characteristics of the specimen.
As discussed earlier in this report the idea is to correlate the spectral transmission characteristics of the specimen to a density value record. The spectral transmission characteristic curves of individual parts are needed to correlate transmission to density. The spectral transmission characteristic curves are also needed to determine the wavelength bands to use for the separation exposures of the specimen. This section of the report text will be on the methods tried and the results obtained from these methods.

Color - Rad Spectro - Colorimeter

The Color - Rad spectro - colorimeter was supplied by Spectral Data Corp. After discussions with Miss Sondra Wenderoth in December of 1973 it was thought that this instrument could extract the spectral characteristics of the specimens parts. This would be by using a fiberoptic probe coupled to the integrating sphere and using the illumination source from the Olympus Microscope. It was felt that the fiberoptic probe on the aerial image plain could extract out the spectral output of the cytoplasm, the nucleus, and the cellular walls.

The Color - Rad was calibrated using the standard instruction manual for the reference lamp and the secondary lamp attached to the unit. After initial tests with the known sources filters of known spectral output were used to determine if the unit was in calibration. After plotting the transmission curves the instrument was determined to be operational. The instrument has a specially designed fiberoptic probe. The fiberoptic probe was attached to the integrating sphere
and placed on the film plain of the photomicrographic head of the Olympus. Recalibration was started for matching the Colo - Rad's reference light source to the illumination output of the tungsten lamp of the Olympus.

Problems arose in the usage of the Color - Rad spectro-colorimeter and the Olympus microscope's illumination source. The design was the usage of the fiberoptic probe on the film plain's aerial image to gather the spectral output of the specimen. Illumination levels for the calibration of the Color-Rad at the wanted magnification of 300X with a lamp voltage of 6 volts were insufficient for the calibration. The main cause seems to be the needed illumination levels to pass through the fiberoptic probe. Because of the limited illumination levels of the Olympus's tungsten lamp 2.4 neutral density units were needed to balance the two lamps illumination output.

Calibration tests proved that the Color - Rad's sensitivity was insufficient for use in recording the spectral transmission characteristics of the microscopic specimen. Repeatability checks over a 5 day period showed + or - values of 25% between the previous days work. Variations were recorded of 15% + or - during daily operations of the Color -Rad. It should also be noted that the area covered by the fiberoptic probe lent to intergration of two or more specimen structures. After these tests were made a alternate plain was needed to extract the spectral transmission characteristics of the specimens components.
Microspectral Photometry

Following discussions with my research advisor Dr. Schumann, an alternate plan was suggested to obtain the spectral transmission characteristic curves of the specimens components. It was suggested to try a method called microspectrophotometry. Microspectrophotometry is used to record the spectral transmission characteristics of areas within a microscopic specimen. The equipment that is used is similar to the proposed design using the Color-Rad's fiberoptic probe on an aerial image plain. The major differences are in the fact that there is a design matching of the component parts of the microspectrophotometer.

The microspectrophotometer is based upon the ideas of a microscope with the addition of a recording spectrophotometer probe in the aerial image plain. Design would allow the selection of areas to be recorded by the spectrophotometers probe in the viewing field of the microscope. The probe design and sensitivity would be matched to the available illumination source that is in the microscope. Because of the small areas covered by the optic probe separation between varying areas of the specimen could be made. Example separations are the nucleus, cellular wall tissue, and the cytoplasm.

This method would yield satisfactory results with the specimens that are under investigation in the experiment. Upon investigation into the availability of the needed equipment the experimental time factor did not permit the usage of a microspectrophotometer to extract the spectral transmission curves of the specimen slides.
Differential Separation of a Biological Specimen.

Through discussions with Dr. J. Baird Head of the Biology department at R.I.T. and several other people in the industry, it was found that a microspectrophotometer was unavailable for my use in the time span remaining for the experiment. After a discussion with Larry Hill, a student in the biology program, a different method was suggested for extracting the spectral transmission characteristics of a specimen. Mr. Hill suggested a method called differential separation.

Differential separation of the biological specimen is the ability for a researcher to extract a singular component out of the specimen. The component part can then be analyzed on an extended range recording spectrophotometer to obtain its spectral transmission characteristics. From the recorded spectral transmission curves proper filters can be fitted to the maximum transmission differences that are between the specimen’s components. The spectral transmission curves are also used to correlate transmission with density values on the separation negatives.

Theory behind the differential separation process states that a specimen’s components are of differing specific gravities, with organic differences in their structure. Through a process of centrifugation the specimen’s components are subjected to varying amounts of force. When a specific force is applied to the specimen for a calibrated period of time the bonds are broken and a specific component is freed into a semi-liquid solution. Therefore differential separation does the following to a biological specimen. Example used is the
extraction of the nucleus from a biological specimen.

The specimen to undergo examination is placed into a test tube. A saline solution is added to the test tube. The specimen and the test tube are placed in a calibrated centrifuge. The centrifuge is set to the required seed and a timer is started. After centrifugation the saline solution is removed from the test tube. There is a fine suspension in the liquid solution, this is the nucleus from the specimen's cellular structure. There are two methods for examination of the spectral transmission characteristic curve after the differential separation process.

The first method for examination is to take the solution and place it on a microscope slide using a smearing technique. Allow the saline solution to dry and only the nucleus's spectral characteristics will be recorded. The slide is then placed in an extended range spectrophotometer and its transmission curve can be recorded.

The second method applies to using the suspended nucleus in the saline solution directly in the recording spectrophotometer. There is a drawback to this method. Before using the test tube and saline combination a transmission record should be made. This characteristic curve is then a reference curve to be subtracted from the results when the test tube contains the specimen component.

Results from several differential separation showed that the wanted region for maximum spectral characteristic traits were in the range of 220nm to approximately 350nm. The spectral range between 350nm to 900nm showed no significant changes in the specimen's transmission. All separations and transmission curves are of unstained specimens.
Spectral Transmission Curves
For the Nucleus and Cytoplasm of a Rabbit's Adrenal Glands.

Cytoplasm = RNA
Nucleus = DNA

Wavelength (\( \lambda \)) in nm.
Figure 1 shows the spectral transmission curves for the differential separations of the nucleus and the cytoplasm of a rabbit's adrenal gland. The curves represented in Figure 1 are an average of three different separations. The data points were taken at 2nm intervals and the averages were calculated and plotted on the transmission versus wavelength graph. The characteristic of the nucleus is represented as the chemical DNA. The characteristic transmission output of DNA is a representative of the nucleus of the rabbit's adrenal gland.

Upon examination of the transmission curve, two peaks are seen: one occurring at 262nm and the second is at 246nm. The main peak is the maximum transmission of the chemical DNA. The second peak is the chemical called associated DNA with its peak at 246nm.

The second curve that was plotted is representative of the cytoplasm's chemical component RNA. The RNA curve was plotted from the average of three differential separations of a rabbit's adrenal glands. The RNA transmission curve peaks at two different wavelengths. The main peak is in the region of 285nm with the second peak at 317nm. The peak transmission at 285nm is the representative frequency of the RNA. The secondary peak at 317nm is for the chemical compound associated RNA.

From a discussion with Miss S. Wenderoth, in January of 1974, a method was proposed to find the maximum wavelength bands and the associated frequencies with the maximum bands. From the transmission curves in Figure 1, the transmission readings are taken and recorded at every 5nm interval. One component is selected to be an averaged normalized curve of the second curve to be plotted against. The
procedure was carried out for the cytoplasm to be the normalized curve. The procedure is as follows, The transmission readings for the cytoplasm are added and the sum is divided by the number of data points taken. This will give the average of the cytoplasm's RNA chemical component. The RNA's transmission average line is plotted on a transmission versus wavelength graph axis. This average is a straight line. The average RNA transmission is then subtracted from the recorded DNA transmission readings. Each point is plotted at the represented wavelength frequency as a plus or minus value from the normalized RNA line. Below is the mathematical formulas that were used.

\[
NT_{RNA} = \frac{375nm}{225nm} \sum_{225nm}^{375nm} RNA(t) / N
\]

\[
NT_{RNA} = \text{Average transmission of the RNA readings.}
\]

\[
RNA(t) = \text{Transmission readings of the RNA at 5nm intervals}
\]

\[
N = \text{Number of data points}
\]

\[
NT_{RNA} = \frac{817.77nm}{31} = 26.377nm = 26.38nm
\]

\[
DNA(t) - NT_{RNA} = TD_{DNA}
\]

\[
DNA(t) = \text{Transmission readings of the DNA at 5nm intervals}
\]

\[
TD_{DNA} = \text{Transmission difference of the NT_{RNA} and the DNA}
\]

The transmission versus wavelength curves for the NT_{RNA} and the TD_{DNA} were plotted. The plotted results are shown in figure 2. The results seen in figure 2 show that the wanted frequency regions
are below 325nm. The wanted frequency bands are 230nm to 240nm with a transmission difference maximum of 24.2%, 245nm to 257nm with a transmission difference of 15.3%, 254nm to 269nm with a transmission difference of 42.3%, 270nm to 300nm with a maximum transmission difference of 16.6%.

Photomicrographic Techniques

This section will be used to discuss the experimental methods used for the photomicrographic reproduction and the tricolor separations of the adrenal glands of the rabbit. The general outline to be considered is (1) Equipment used and its limitations, (2) The tricolor separation exposures, (3) Sensitometry of the tricolor separations, (4) The results of the black and white tricolor separations.

(1) Photographic Equipment

A.) Olympus model FH microscope, Built in illuminator with a 1.25 N.A., TE - 11 variable voltage power supply with voltage meter, trinocular head w/H.E.P., WF 10X, P 10X for Photomicrographic work Objectives; 4X, 10X, 40X, 100X (oil), Stage - graduated mechanical rotatable 360°

B.) Bausch and Lomb model L stand, Photomicrographic work at 4" x 5" film format.

C.) Polariod MP4 film back adapted for use on the Bausch and Lomb model L stand.

D.) Films;

a.) Kodak Plus X Pan Professional No 4147 4" x 5" format
b.) Kodak High Speed Infrared No 4143 4" x5"format
c.) Kodak Ektachrome Photomicrographic 135mm format
E.) Filters;
   a.) Kodak Wratten gelation 3" x 3" No. 25 - red
   b.) Kodak Wratten gelation 3" x 3" No. 58 - green
   c.) Kodak Wratten gelation 3" x 3" No. 47B - blue
   d.) Kodak Wratten gelation 3" x 3" No. 29 - infrared
   e.) Kodak Wratten gelation 3" x 3" No. 89B - infrared

F.) Processing;
   a.) Kodak D - 76 for the black and white films
   b.) Kodak E - 4 processing for the photomicrographic film

The above list of equipment was used to carry out most of the photographic reproduction of the experiment. By the results seen on pages 20 and 21 figures 1 and 2 the wanted spectral regions are below the optical capabilities of the Olympus Microscope. The needed equipment for the optics of the experiment are ether quartz of floride designed. At this point I would refer the reader to the text of R. P. Loveland., Photomicrography A Complete Treatise, John Wiley and Son Inc. New York, New York.,1970 Chapter 14 pages 642 - 686. This text will give a good reference to the needed optical materials for work in the near and far ultraviolet region of the spectrum.

(2) Black and White Films Response to the Tricolor Separation Filters

Photographic speeds are based on the response of photographic materials to broad-band radiation often to "white" light. The variation in sensitivity with wavelength is termed spectral sensitivity, and is a very important characteristic of a photographic material.
Based upon these statements a test was made to determine if using the tricolor separation filtered illumination would alter the characteristic photographic response of Kodak Plus X Pan Professional No.4147.

Qualitative measurements of spectral sensitivity, useful for comparative purposes, may be made by making sensitometric exposures through filters and determining the relative sensitivity, (1) by the exposure difference for visually matched densities, or (2) from plotted D log E curves using the exposure for a given density.\(^{18}\) The second method stated above was used for the sensitometric evaluation. There were four sensitometric strips exposed and processed. The exposures were made in a Kodak model 101 sensitometer in the following series order, white light illumination, red No. 25 Kodak Wratten gelation filter, green No. 58 Kodak Wratten gelation filter, blue No. 47B Kodak Wratten gelation filter. The sensitometric strips were processed in Kodak D - 76 for 6 minutes at 68°C. All the strips were processed at the same time. Agitation was by the tray rock method. The densities were recorded from a MacBeth model TD 102 densitometer.

The sensitometric curves were plotted with consideration to the band width and the filters absorption characteristics in the spectral band. There was also consideration taken for the filter factors from the separation filters used in the exposures. The numbers for the filter factors are No. 25 red - 4, No. 58 green - 8, and No. 47B blue - 12. The log of the filter factor numbers was taken and subtracted from the log of the exposure for each step of the 21-step step tablet. The resultant sensitometric curves are plotted and the results are seen
Sensitometric Variation
From Tri-color Separation Filters on Kodak
Plus-X PAN Professional
No. 4147

Density

Log Exposure

25 Red $Y = 0.60$
472 Blue $X = 0.62$
58 Green $Y = 0.64$
911 White $Y = 0.68$
in figure 3 on page 27. Figure 3 shows the sensitometric relationships as density verses the log exposure for the four exposures. Note the slight differences in the gamma values of the four curves. The gamma values for the red exposure is \( \gamma = 0.60 \), for the blue exposure is \( \gamma = 0.62 \) for the green exposure \( \gamma = 0.64 \), and for the white light exposure \( \gamma = 0.68 \).

The maximum difference in the gamma values is the 0.08 value for the red and the white light exposures. The differences in the gamma values for each of the exposures is 0.02. It is felt that this difference is not significant for the exposures and processing to be used in the experiment.

The Tricolor Separation Negatives

The equipment used in the photomicrographic exposures was discussed earlier in the report under the section on page 24 the Photographic equipment section. The optical schematic diagram for the filter placement is found in figure 4 on page 29 of the report.

The optical configuration and the exposures used in the experiment are recorded in table 1 found on page 30. The table states the exposure times and processing methods used in the experiment.
Figure 4
Table 1

Expoure Series on Plus X Pan Professional No. 4147.

Filters:

<table>
<thead>
<tr>
<th>None</th>
<th>Exposure times - 1, 2, 4, 8, seconds</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10X FK occular - N.A. .68</td>
</tr>
<tr>
<td></td>
<td>4X Plane Objective - N.A. .10</td>
</tr>
<tr>
<td></td>
<td>1.25 N.A. Abbe Condenser substage</td>
</tr>
<tr>
<td></td>
<td>4&quot; x 5&quot; film plain 28cm from the FK occular</td>
</tr>
<tr>
<td></td>
<td>Processing Kodak D-76, 68°F, 6min, tray rock</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>47B</th>
<th>Exposure times - 4, 8., 16, 32 seconds</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>10 FK occular - N.A. .68</td>
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<tr>
<td></td>
<td>4X Plane Objective - N.A. .10</td>
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<tr>
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<td>4&quot; x 5&quot; film plain 28cm from the FK occular</td>
</tr>
<tr>
<td></td>
<td>Processing Kodak D76, 68°F, 6 min., tray rock</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>25</th>
<th>Exposure times - 1, 2, 4, 8, seconds</th>
</tr>
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<tr>
<td></td>
<td>10X FK occular - N.A. .68</td>
</tr>
<tr>
<td></td>
<td>4X Plane Objective - N.A. .10</td>
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<tr>
<td></td>
<td>4&quot; x 5&quot; film plain 28cm from the FK occular</td>
</tr>
<tr>
<td></td>
<td>Processing Kodak D-76, 68°F, 6 min., tray rock</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>58</th>
<th>Exposure times - 1, 2, 4, 8 seconds</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>10X FK occular - N.A. .68</td>
</tr>
<tr>
<td></td>
<td>4X Plane Objective - N.A. .10</td>
</tr>
<tr>
<td></td>
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<td>4&quot; x 5&quot; film plain 28cm from the FK occular</td>
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<tr>
<td></td>
<td>Processing Kodak D-76, 68°F, 6 min., tray rock</td>
</tr>
</tbody>
</table>
Tricolor Separation Results

In photomicrography using black and white films, filters are used for control of the image contrast. The staining colors used were hemotoxylin as a deep purple and the erosin counter stain as a red. Filtration can be used for a maximum in contrast control. If maximum contrast control is desired between a colored specimen and the background, the filter should absorb the specimen's color completely. For the stained specimen this can easily be found but with the nonstained specimen a method of darkfield illumination was needed. The darkfield illumination method of exposure cannot be filtered. Therefore only the stained specimen was subjected to the separation exposures. The results of the exposures are shown in figures 6, 7, 8, with the white light exposure shown in figure 5.

SUMMARY and RECOMMENDATIONS

As seen throughout the report the initial outline, it is not possible at this time for a photographic means to create a visual false coloring in a nonstained specimen. In summary the results do show that experimentation can be carried out to attempt a further try at a possible system if the needed optics are used.

Recommendations are to find the needed photomicrographic equipment that will resolve down to the spectral regions of 220 nm. This is in the far ultraviolet and the needed filtration to correspond to the transmission output of the specimen.
Figure 5
White Light exposure 1 sec.
Polycontrast No. 2, 15 sec.
300x magnification
stained slide No. 5

Figure 6
Blue Filter exposure 4 sec.
Polycontrast No. 2, 25 sec.
300x magnification
stained slide No. 5

Figure 7
Red Filter exposure 2 sec.
Polycontrast No. 2, 12 sec.
300x magnification
stained slide No. 5

Figure 8
Green Filter exposure 2 sec.
Poly contrast No. 2, 15 sec.
300x magnification
stained slide No. 5
FOOTNOTES


2.) Ibid
3.) Ibid page 1023
4.) Ibid page 1024
5.) Ibid

6.) Kodak Infrared Films, Kodak Technical Publications No N-17 Eastman Kodak Company, Rochester, New York page 3


9.) Ibid
10.) Ibid
11.) Ibid
12.) Ibid
13.) Yost and Wenderworth page 1027
14.) Ibid
15.) Ibid


18.) Ibid page 274

20.) Ibid
BIBLIOGRAPHY


Bergner, J., E. Glibke, and W. Mehliss., Practical Photomicrography. (tr. from German), Focal Press, N.Y., N.Y. 1966

Braver, Alfred., Laboratory Directions for Histological Technique. Burgess Publishing Co., Minneapolis, Minn. 4th printing, 1961


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F-5, N-1, M-28, E-3, M-29, N-12A, N-12B, N-17, P-2, P-315, N-9, Eastman Kodak Company, Rochester, New York 14650

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