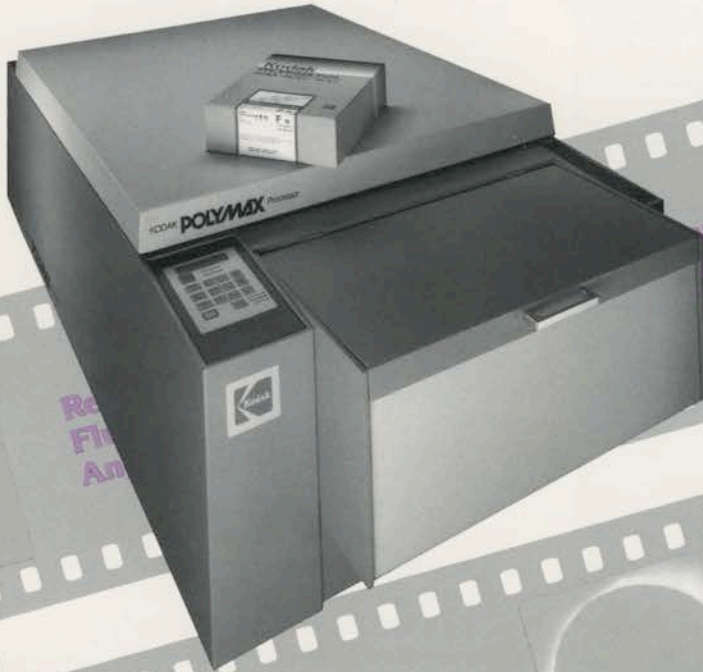


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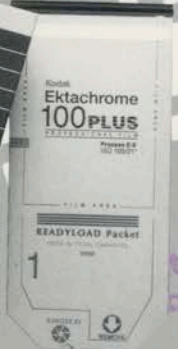


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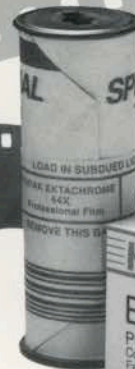
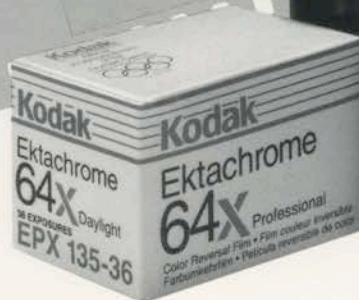
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Retinal  
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Tone  
Reproduction in  
Photomicrography

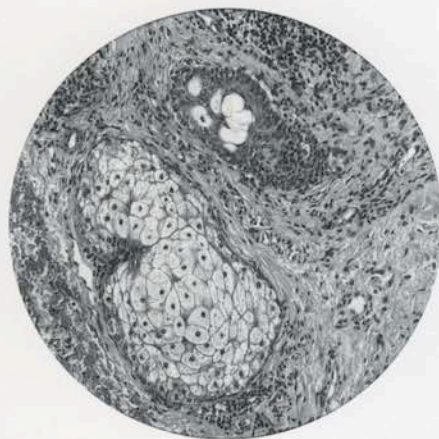
Tone  
Reproduction in  
Photomicrography



ISSUE NO. 1, 1991

## Precise Black-and-White Tone Reproduction in Photomicrography

by Michael Peres, RBP, and Claudia Murphy  
School of Photographic Arts & Sciences  
Rochester Institute of Technology  
Rochester, New York 14623



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### Introduction

Producing black-and-white photomicrographic negatives has traditionally been a task that, in many instances, required a trial-and-error approach. Such methods left many experienced photomicrographers frustrated by the lack of quality in their work. This has included the authors who have struggled to produce quality negatives necessary for accurate tone reproduction.

In producing color transparencies, the photographic problem is to get accurate exposure to insure color saturation. When the transparency film has been properly exposed, all the various stains and counterstains are easily distinguishable. Even the subtlest shades are discernible because of the stain's color contrast. Blue is easily differentiated from lavender. Red is easily distinguished from magenta, and so on.

In black-and-white photomicrography, color contrast is absent. A shade of green may reproduce as the same shade of gray as a red in the final print because of the minimal density difference between the stains when rendered as monochromes. In most instances, it is difficult to distinguish quickly the various cell types with no color reference.

The use of contrast-producing filters is the quickest method to get increased separation of values in the black-and-white negative. If more separation is needed between the various densities of hemotoxylin and eosin (H & E) stains, the use of a KODAK WRATTEN Filter No. 11 will assist in getting a good degree of separation. This assists the tone reproduction process greatly, but additional testing may be necessary to get a better negative. Unless you handle routine specimens all the time, many situations may require several contrast and film tests to get the best negatives. (For more information on the role of filters in photomicrography, see *Photography Through the Microscope*, KODAK Publication No. P-2.)

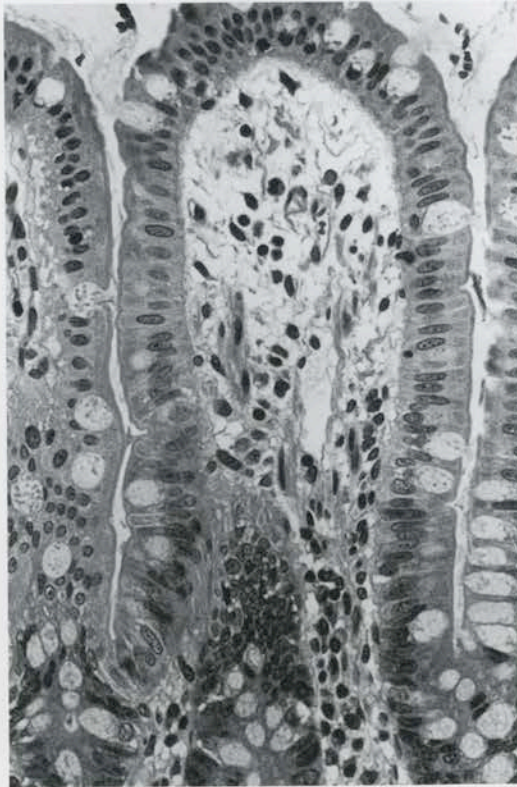
The making of a good black-and-white negative in photomicrography usually requires some initial testing to determine the contrast requirements of the specimen.

When photographing through the microscope with brightfield technique, there is a scene luminance range that could be approximately 16:1 with an average H & E stained subject. This ratio refers to the separation between the useful high-light and shadow exposure—only about three-and-one-half to four *f*-stops. In other words, this is a very low contrast situation, which will result in a very flat negative. This results in poor tone reproduction in the final print. Each step in the process affects the final result.

A typical photographic emulsion can routinely manage a luminance range of 160:1 or more than seven *f*-stops. Hence, with a typical photomicrographic situation in a bright-field microscope, there isn't a good match of inherent film contrast to specimen contrast. For this reason, a photomicrographer often chooses overdevelopment as a routine procedure to spread out the values. For example, the selection of KODAK T-MAX 100 Professional Film and overdevelopment might be an appropriate choice for a typical hemotoxylin and eosin (H & E) stain.

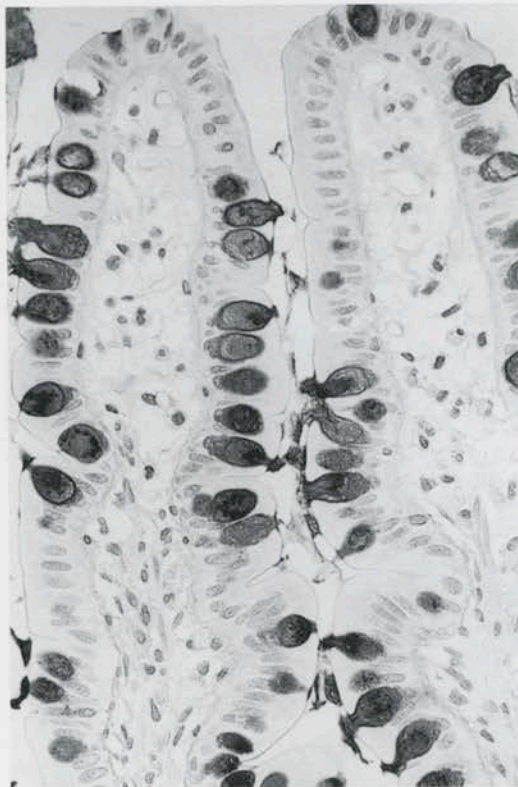
Does this approach always work and how good are the negatives? The user usually evaluates the results for internal image separation. After inspecting the negative, the photomicrographer can determine if a better photographic strategy would yield a good negative. The strategies, based on the specimen contrast requirements, could include

- Choosing an alternate film, e.g. KODAK Technical Pan Film or KODAK T-MAX 100 Professional Film.
- Choosing alternate developers, e.g. KODAK Developer D-76 or KODAK MICRODOL-X Developer.
- Adjusting contrast index (CI) by varying development time.



Application of the system described here resulted in these images.

(A) The slide on the top was a 3-micrometre cross section of stomach stained with H&E. The magnification at the print was X470. The negative was made, using a KODAK WRATTEN Gelatin Filter, No. 11 (green), on KODAK Technical Pan Film rated at an EI 25 and developed to a CI of 1.4 in KODAK HC-110 Developer (F); the negative was printed on a grade 2 paper.



(B) This thin section (2 micrometres) of stomach was stained with light alcohol blue. The magnification at the print was X470. Technical Pan Film was rated at EI 25 and developed to a CI of 1.65 in HC-110 Developer (F). The print was made on grade 3 paper.

Photography by Wm. Scott Lawrence

## Background

The Laboratory for Photomicrography/Photomacrography at the Rochester Institute of Technology undertook development of a system that allows a photomicrographer to have, ready at hand, information for film selection and development strategies based on subject stain, staining density, and specimen thickness.

The proposed system needed to utilize an at-the-film-plane metering technique and have a pre-defined relationship of specimen contrast requirements to film and developer choice.

Two emulsions were selected to evaluate, because in all probability they would serve most photomicrographic situations. These two films were KODAK Technical Pan Film and KODAK T-MAX 100 Professional Film. The first phase of research was to test the films for their specific exposure indices and contrast indices. (See "Sensitometric Evaluation of KODAK Technical Pan Film and KODAK T-MAX 100 Professional Film Using a Wide Range of Developments," *TECH BITS*, Issue No. 2, 1990.) The next phase of the research was to devise a means for matching the log exposures of the subject to the film's useful exposure latitude. (See "A System for Exposure Determination and Tone Reproduction Control," *TECH BITS*, Issue No. 3, 1990.) The final phase of the research was to match a given film's contrast index to a specimen of known thickness and stain and to evaluate the subsequent tone reproduction.

## The Hypothesis

Might there be an easier method to get good tonal separation other than the random selection of films and developers? Is there a relationship between stains or staining density or specimen thickness and final image contrast? If there is a relationship, what is the easiest way to determine and use it?

In evaluating these questions, several relationships were indicated that would need to be proven. A specimen that is thinner would probably be less contrasty and would warrant an increased contrast treatment. A specimen that is thicker will be more contrasty and would warrant a reduced contrast treatment. A specimen that is stained heavily would be contrasty and thus require a flatter treatment than a specimen that is weakly stained. In making these projections it became apparent that some stains are more contrasty than others and that each stain might require a different contrast.

## The Strategy

With a working hypothesis and some interesting relationships to be considered, our next step was to prove the hypothesis and to propose a new approach for generating useful black-and-white tone reproduction in photography through the microscope.

This proposed system would require two integral steps in the process. The first would utilize a new metering technique and the second would involve selecting a film-developer contrast index, based on specimen characteristics, that would allow for accurate tone reproduction with minimal effort.

## The Metering Technique

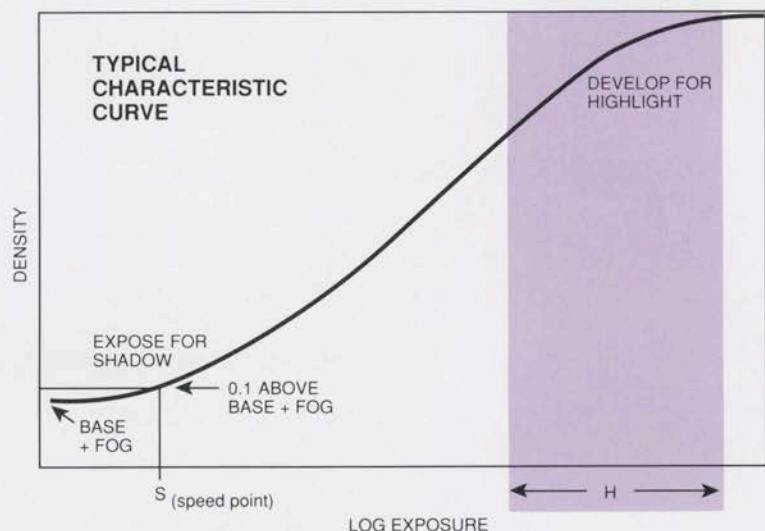
As it evolved, the system would be based on the metering of the clear field without the specimen (one of the few constants in the photomicrographic system) and would produce a negative with a constant highlight density. The metering method, originally proposed by Dr. Roger Loveland, RBP, FBPA, and further refined by Jack Holm, would allow for precise exposure determination regardless of the specimen photographed. The film and developer combination would subsequently be chosen based on the specimen requirements. The film could be exposed precisely by placing the correct exposure in the highlight region of the material, allowing the subsequent exposures to fall below. This technique is a common practice when working with reversal materials.

The reasoning behind clear-field metering is simple because of a problem specific to photomicrography and one not found in traditional photography. When making exposure determinations with a traditional photographic system, a photographer usually bases the exposure of the material on the shadow values of the scene. The shadow metering is designed to give the material the correct exposure. Giving a photographic emulsion the right exposure will allow the material's grain structure to remain tight and be effectively used. The result will be good photographic resolution which is important for fine detail recording in photomicrography. Overexposure to a material conversely will cause a loss of fine detail because of increased granularity as well as a loss of resolution. It is then obvious that placing the minimal exposure on the material is critical to the photographic process.

In traditional photographic situations, it becomes very easy to make shadow meter readings that allow for exposure to be placed near the minimum density of a photographic material. The photographer simply takes an exposure-meter reading based on reflective measurements in the scene that are determined to be in the shadow area or an area of low reflectance.

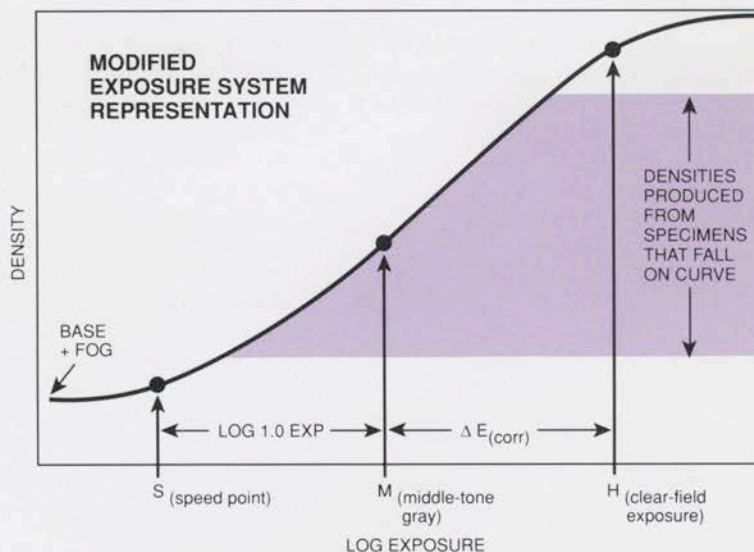
Because a light meter turns all readings to 18-percent gray, the photographer must correct the reading based on how far into the shadow the reading was made. This research is not about proper exposure placement in traditional photographic situations but rather about exposure determination through the microscope.

Let us review for a moment the graphic representation of film response as demonstrated by a D-log E curve. The results (densities) caused by a series of exposures are plotted against the log exposure values. The densities produced from a shadow exposure would be located on the toe region of a curve. This is the region of the



A typical film characteristic curve reflects traditional exposure and development strategy with exposures placed in the shadow region of the response curve and the highlights developed to the shoulder of the curve. The inertia or speed point of the material is located 0.1 density units above base plus fog.

This characteristic curve demonstrates the application of the exposure and development strategy described in this article. The speed point lies at point S. Middle gray (18 percent) response is at point M. Location H identifies the highlight exposure required to produce an adequate clear-field density. The slope of this characteristic curve depends upon the speed point and contrast index choice made prior to exposing the negative. For guidance on moving the exposure from 18-percent gray to the proper highlight value, refer to Table 1 that accompanied the article by Jack Holm, "A System for Exposure Determination and Tone Reproduction Control in Black-and-White Photography," *TECH BITS*, Issue No. 3, 1990. Use of this table is necessary for choosing the best EI-CI combination.



plot where the material just begins to respond to light and produce densities. There is a midline and a shoulder where the highlight regions reside.

Many contemporary strategies have been created for those who are concerned with proper placement of shadow exposure and development for highlight brightness. What generally results is a reasonably good negative that manages the scene luminance range effectively. A very popular use of the technique of exposure based on exposure for the shadows and development for the highlights is the Zone System made popular by Ansel Adams and others.

Unfortunately metering or accessing shadow information is not an easy task for the photomicrographer. It is, for all practical purposes, impossible to meter the shadow of a thin section accurately. Consider a routine microscopic preparation. Located in the shadow regions are the nuclei of most cells and these will be a few microns in diameter at best. These regions tend to be, on the whole, rather small; so finding a region large enough to cover a metering cell is not a good probability. (We are not aware of any metering systems, even the smallest spot meters, that are capable of metering one nucleus.) Accessing important shadow exposure values becomes a real problem if the photographer wants to base the exposure on it.

A very common solution to the metering problem that produces an acceptable result is average metering. Just meter the whole field of view, rate the film normally based on the manufacturer's recommendations, and bracket around that value. This is adequate for many cases but does not allow for precise control of exposure. This, in turn, does not allow for precise control of contrast and tone reproduction. The results are adequate but often not great.

How can one get better-than-average exposure information? The first step is to set up the photomicroscope with Kohler illumination. The photomicrographer can then use the clear-field method to determine precise exposure information. In any brightfield microscope, there exists a clear field or region in the field of view where there is total light transmission. This clear field can easily be metered (at proper lamp voltage for photography and with no specimen) and used to produce a specific density on the photographic material. Having established the field of interest, the photomicrographer can record the coordinates of the field and then move the specimen to a region where only the clear field is visible. The user can then take a meter reading, lock in that exposure, and move back to the field of interest. (Using this exposure method will produce a good negative of proper highlight density.)

The system depends upon an integrated metering system that is capable of determining accurate shutter speed information at the film plane. At-the-film-plane metering is essential so that there is no light loss from the camera

system. When light is relayed in a photographic camera system, some of the light may be lost due to system effects. If the metering is done anywhere other than at the film plane, correction factors will need to be applied so as to avoid exposure errors.

Since a reflected light metering system translates all the illumination it reads to produce 18-percent gray, the photographer must adjust the exposure to produce an accurate highlight value. (Using the clear-field reading alone would result in an improperly exposed negative. For this reason, the exposure must be adjusted by an amount to be determined either mathematically or by testing. A more accurate method is demonstrated in the article by Jack Holm, "A System for Exposure Determination and Tone Reproduction Control in Black-and-White Photography," *TECH BITS*, Issue No. 3, 1990. Setting the meter's sensitivity to the ascribed exposure index (EI) from the tables with that article will nullify this problem and the exposure reading will produce the proper exposure for the material.)

What is produced will be an appropriate highlight density on the film that will reproduce on a printing paper close to paper white using a grade 2 paper. This will allow the remainder of the densities to fall on the region of the film's response where adequate separation between the various tonalities of a specimen can be best reproduced. The region on film where the best separation of values can occur is the straight line portion of the characteristic curve.

## The Relationship of Specimen and Contrast Index

The next part of the project entailed testing the relationships of specimen characteristics to contrast indices of various film-developer combinations. A typical H & E specimen 6  $\mu\text{m}$  thick is best photographed under conditions that produce an approximate contrast index of 0.8. The rest of the research involved making other projections and testing the results.

The characteristics that we have identified as playing a role in this process are

- Stain contrast
- Stain density
- Specimen thickness

The user can evaluate each of these situations, although somewhat subjectively, to allow the selection of the appropriate film CI. The photomicrographer must identify the stain, consulting the technologist who prepared it if it is unknown. It is important to know the stain characteristics if the system is to work.

For comparative purposes, consider that a silver stain is much contrastier than an H & E stain and that PAS is not as contrasty as triple connective stain. The determination of what the contrast requirements of a stain are, so as to choose the best negative CI, is an important consideration for the system to work. The hypothesis is that the contrastier the stain was visually, the lower the CI needed to get a good negative.

The next specimen characteristic considered to play a role in tone reproduction is staining density. As with any chemical process, the activity of the stain can be quite varied based on age of the stain, the pH of the stain, or the activity of the process. This range of activity results in stains that can either be rich and full all the way

through the specimen or washed out. The hypothesis here is that the richer, fuller the stain, the less the contrast requirement of the film. The weaker the stain, the more the contrast needed to provide a good negative.

The last variable and specimen characteristic that plays a role in tone reproduction is specimen thickness. The thicker the tissue the denser the tissue. If the tissue is denser, it stops more light and consequently can be, in some cases, contrastier than thinner sections. Based on the equipment used and the supporting material, e.g., paraffin versus resins, a specimen may range from 2–3  $\mu\text{m}$  up through 12–13  $\mu\text{m}$  in thickness. It is obvious that this range has a direct effect on specimen contrast and so plays a role in which negative CI to choose.

Specimen thicknesses are usually available from the histotechnologist who prepared the tissue, but if not, the photomicrographer can determine the thickness. The first step is to focus on the top of the tissue and look at the micrometer scale on the fine-focus knob. (The fine-focus assembly is calibrated in optical micrometers.) Record the value. The next step is to focus the specimen at the bottom of the tissue and record the micrometer reading. By subtracting the two values, the photomicrographer can determine specimen thickness if the tissue is suspended in air. If it is not in air and is embedded in a mounting medium such as permount or Canada balsam, multiply the difference of the two values by the refractive index of the medium. (For more information on this process consult *Photography Through the Microscope*, KODAK Publication No. P-2.)

## Using the System

Using the system is quite simple once the photomicrographer understands the concepts of what it will provide. Below is the step-by-step procedure for applying the system.

1. Set up Kohler illumination and locate the field to be photographed.
2. Determine the contrast requirements of the subject either by using the tables provided or by visual comparison to a known.
3. Select a film and developer combination that will yield the desired CI. Use either information from *KODAK Professional Black-and-White Films*, KODAK Publication No. F-5, or the film tables from *TECH BITS*, Issue No. 3, 1990.
4. Determine the film sensitivity (EI) of the emulsion based on the treatment. Locate this with the contrast index information.
5. Apply the correction factor for clear-field metering. Locate this in the tables or add 2.5 stops to the exposure index.
6. Set the at-the-film-plane metering system with the EI value thus determined.
7. Record the coordinates of the tissue and move the tissue out of the field of view so that only a clear field is present.
8. Take a meter reading, lock it in, and return to the area of interest in the specimen.
9. Make the exposure and process the film to achieve the CI determined in step 2.
10. Make a print from the resulting black-and-white negative. The negative should print easily on a grade 2 paper.

## Results to Date

Below is a listing of the results of our investigations to date. Much work lies ahead to examine further the hundreds of stains and combinations of stains utilized in the biological world. The results so far substantiate the original premise of the work. There is strong relationship between stain, stain concentration, and thickness as they influence tone reproduction.

KODAK Technical Pan Film and KODAK T-MAX 100 Professional Film can produce a tremendous range of contrasts. There exists the possibility of going from a CI of 0.19 in the lowest contrast situation to a CI of 3.0. By determining the contrast requirements of the specimen and choosing a film and developer combination that allows for accurate tone reproduction, you can apply the system effectively. Keep good records and the system will be an invaluable tool in producing quality tone reproduction from your black-and-white photomicrographic negatives.

In developing the system, we used 35 mm roll film. The process of use is much more critical than the format. The methods would also apply, with some testing, to 4 x 5-inch negatives. In some instances this will actually be easier.

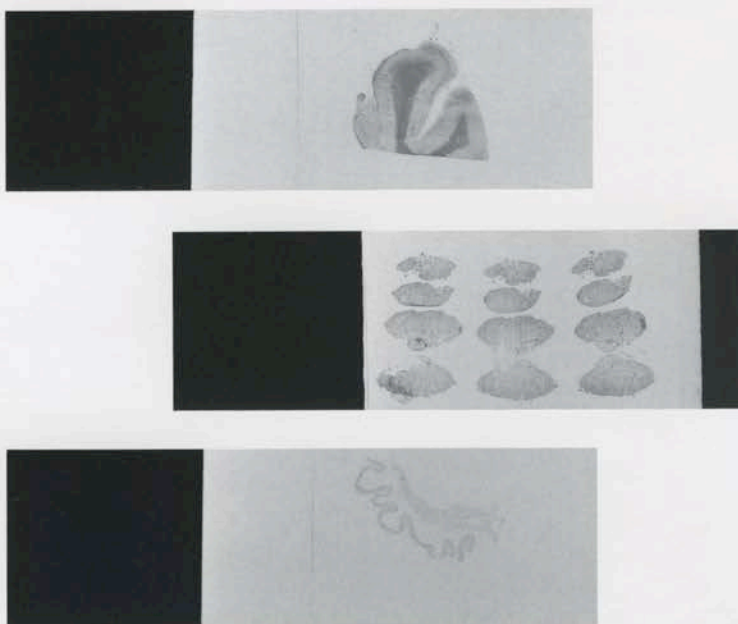
Applying the system will require attention to detail. It does require some time to set up, move the specimen around, meter, determine the desired film CI, and determine the resulting EI. This process may seem a bit cumbersome at first, but it is actually faster than having to generate new negatives if the initial result is poor.

Another problem may arise if you regularly use 36-exposure rolls of 135 film. What happens when specimens of totally different treatments are on the same roll? If this is a regular occurrence, you may wish to load short lengths of film from bulk rolls. This will allow for individualized treatments of each subject and not waste material.

Working with oil can also present difficulties. If there is no clear field close by the field to be photographed, it may be next to impossible to use the system. Finding a field under oil might be very slow and time consuming. Moving the field to take a meter reading and relocating the specimen may make the system impractical.

A problem exists with automatic metering systems having no memory-locking capabilities. If the photomicrographic camera system you are using has no ability to take a reading and store it in memory for future use, the system will not function for you. The tissue, you will remember, must be moved to take a reading. If you read the clear field and cannot store that reading, you get only an average reading.

The ultimate goal of the system is to insure accurate tone reproduction. It does provide that—with some potential problems to overcome. The goal of the project was to provide the photomicrographer with more control in black-and-white photomicrography. You can let us know of your impressions of this strategy and your experiences by writing us at our RIT address.



*This series of microscope thin sections demonstrates the range of subject contrasts that a photomicrographer may encounter. They are, from top to bottom, (1) PAS stain, 6 micrometres thick; (2) H&E, 5 micrometres, average stain density; (3) Alcohol blue, lightly stained.*



**Suggested Contrast Index Requirements  
Based on Specimen Characteristics**

**Various Stains and Thicknesses Evaluated**

Stain	Concentration	Thickness	CI Requirements	WRATTEN Filter
H & E	Normal	9 $\mu\text{m}$	0.8	11
Alcohol Blue	Normal	3 $\mu\text{m}$	1.5	—
H & E	Normal	3 $\mu\text{m}$	1.4	11
PAS	Normal	2 $\mu\text{m}$	1.2	—
Movat	Normal	2 $\mu\text{m}$	1.4	—

**H & E Stain Thickness Comparisons**

Stain	Concentration	Thickness	CI Requirements	WRATTEN Filter
H & E	Normal	4 $\mu\text{m}$	1.4	11
H & E	Normal	5 $\mu\text{m}$	1.0	11
H & E	Normal	6 $\mu\text{m}$	0.9	11
H & E	Normal	7 $\mu\text{m}$	0.9	11
H & E	Normal	8 $\mu\text{m}$	0.8	11

**H & E Stain Density Comparisons**

Stain	Concentration		Thickness	CI Requirements	WRATTEN Filter
	(Hematoxylin)	(Eosin)			
H & E	Heavy	Light	5 $\mu\text{m}$	1.0	11
H & E	Heavy	Normal	5 $\mu\text{m}$	1.0	11
H & E	Heavy	Heavy	5 $\mu\text{m}$	1.0	11
H & E	Normal	Light	5 $\mu\text{m}$	1.0	11
H & E	Normal	Normal	5 $\mu\text{m}$	1.2	11
H & E	Normal	Heavy	5 $\mu\text{m}$	1.2	11
H & E	Light	Light	5 $\mu\text{m}$	1.0	11

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